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Title:

MODIFIED PROTEIN KINASE A-SPECIFIC OLIGONUCLEOTIDES AND METHODS OF THEIR USE

Inventor:

Sudhir Agrawal

Assignee:

HYBRIDON, INC.
A Corporation of the State of Delaware

MODIFIED PROTEIN KINASE A-SPECIFIC OLIGONUCLEOTIDES AND METHODS OF THEIR USE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a non-provisional continuation-in-part application claiming priority from U.S.S.N. 60/103,098, filed on October 5, 1998, and from U.S.S.N. 09/022,965, filed on February 12, 1998, which is a continuation-in-part application of U.S.S.N. 08/532,979 filed September 22, 1995.

FIELD OF THE INVENTION

The present invention relates to cancer therapy. More specifically, the present invention relates to the inhibition of the proliferation of cancer cells using modified antisense oligonucleotides complementary to nucleic acid encoding the protein kinase A RI_{α} subunit.

BACKGROUND OF THE INVENTION

The development of effective cancer therapies has been a major focus of biomedical research. Surgical procedures have been developed and used to treat patients whose tumors are confined to particular anatomical sites. However, at presentation, only about 25% of patients have tumors that are truly confined and amenable to surgical treatment alone (Slapak et al. in Harrison's Principles of Internal Medicine (Isselbacher et al., eds.) McGraw-Hill, Inc., NY (1994) pp. 1826-1850). Radiation therapy, like surgery, is a local modality whose usefulness in the treatment of cancer depends to a large extent

on the inherent radiosensitivity of the tumor and its adjacent normal tissues. However, radiation therapy is associated with both acute toxicity and long term sequelae. Furthermore, radiation therapy is known to be mutagenic, carcinogenic, and teratogenic (Slapak et al., ibid.).

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Systemic chemotherapy alone or in combination with surgery and/or radiation therapy is currently the primary treatment available for disseminated malignancies. However, conventional chemotherapeutic agents which either block enzymatic pathways or randomly interact with DNA irrespective of the cell phenotype, lack specificity for killing neoplastic cells. Thus, systemic toxicity often results from standard cytotoxic chemotherapy. More recently, the development of agents that block replication, transcription, or translation in transformed cells, and at the same time defeat the ability of cells to become resistant, has been the goal of many approaches to chemotherapy.

One strategy is to down regulate the expression of a gene associated with the neoplastic phenotype in a cell. A technique for turning off a single activated gene is the use of antisense oligodeoxynucleotides and their analogues for inhibition of gene expression (Zamecnik et al. (1978) *Proc. Natl. Acad. Sci. (USA)* **75:**280-284). An antisense oligonucleotide targeted at a gene involved in the neoplastic cell growth should specifically interfere only with the expression of that gene, resulting in arrest of

cancer cell growth. The ability to specifically block or down-regulate expression of such genes provides a powerful tool to explore the molecular basis of normal growth regulation, as well as the opportunity for therapeutic intervention (see, e.g., Cho-Chung (1993) *Curr. Opin. Thera. Patents* **3:**1737-1750). The identification of genes that confer a growth advantage to neoplastic cells as well as other genes causally related to cancer and the understanding of the genetic mechanism(s) responsible for their activation makes the antisense approach to cancer treatment possible.

One such gene encodes the RI_{α} subunit of cyclic AMP (cAMP)-dependent protein kinase A (PKA) (Krebs (1972) *Curr. Topics Cell. Regul.* **5:**99-133). Protein kinase is bound by cAMP, which is thought to have a role in the control of cell proliferation and differentiation (see, e.g., Cho-Chung (1980) *J. Cyclic Nucleotide Res.* **6:**163-167). There are two types of PKA, type I (PKA-I) and type II (PKA-II), both of which share a common C subunit but each containing distinct R subunits, RI and RII, respectively (Beebe et al. in *The Enzymes: Control by Phosphorylation*, 17(A):43-111 (Academic, New York, 1986). The R subunit isoforms differ in tissue distribution (Øyen et al. (1988) *FEBS Lett.* **229:**391-394; Clegg et al. (1988) *Proc. Natl. Acad. Sci. (USA)* **85:**3703-3707) and in biochemical properties (Beebe et al. in *The Enzymes: Control by Phosphorylation*, 17(A):43-111 (Academic Press, NY, 1986); Cadd et al. (1990) *J. Biol. Chem.*

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265:19502-19506). The two general isoforms of the R subunit also differ in their subcellular localization: RI is found throughout the cytoplasm; whereas RI localizes to nuclei, nucleoli, Golgi apparatus and the microtubule-organizing center (see, e.g., Lohmann in *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*, 18:63-117 (Raven, New York, 1984; and Nigg et al. (1985) *Cell* **41:**1039-1051).

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cmT5

An increase in the level of RI_α expression has been demonstrated in human cancer cell lines and in primary tumors, as compared with normal counterparts, in cells after transformation with the Ki-*ras* oncogene or transforming growth factor-α, and upon stimulation of cell growth with granulocyte-macrophage colony-stimulating factor (GM-CSF) or phorbol esters (Lohmann in *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*, **18:**63-117 (Raven, New York, 1984); and Cho-Chung (1990) *Cancer Res.* 50:7093-7100). Conversely, a decrease in the expression of RI_α has been correlated with growth inhibition induced by site-selective cAMP analogs in a broad spectrum of human cancer cell lines (Cho-Chung (1990) *Cancer Res.* 50:7093-7100). It has also been determined that the expression of RI/PKA-I and RII/PKA-II has an inverse relationship during ontogenic development and cell differentiation (Lohmann in *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*, Vol. 18, 63-117 (Raven, New York, 1984); Cho-Chung (1990) *Cancer Res.* 50:7093-7100). The RI_α subunit of PKA has

thus been hypothesized to be an ontogenic growth-inducing protein whose constitutive expression disrupts normal ontogenic processes, resulting in a pathogenic outgrowth, such as malignancy (Nesterova et al. (1995) *Nature Medicine* **1**:528-533).

Antisense oligonucleotides directed to the RI_{α} gene have been prepared. U.S. Patent No. 5,271,941 describes phosphodiester-linked antisense oligonucleotides complementary to a region of the first 100 N-terminal amino acids of RI_{α} which inhibit the expression of RIα in leukemia cells *in vitro*. In addition, antisense phosphorothioate oligodeoxynucleotides corresponding to the N-terminal 8-13 codons of the RI_{α} gene was found to reduced *in vivo* tumor growth in nude mice (Nesterova et al. (1995) *Nature Med.* **1**:528-533).

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Unfortunately, problems have been encountered with the use of phosphodiester-linked (PO) oligonucleotides and some phosphorothioate-linked (PS) oligonucleotides. It is known that nucleases in the serum readily degrade PO oligonucleotides. Replacement of the phosphodiester internucleotide linkages with phosphorothioate internucleotide linkages has been shown to stabilize oligonucleotides in cells, cell extracts, serum, and other nuclease-containing solutions (see, e.g., Bacon et al. (1990) *Biochem. Biophys. Meth.* **20**:259) as well as *in vivo* (Iversen (1993) *Antisense Research and Application* (Crooke, ed) CRC Press, 461). However, some PS oligonucleotides have been found

to exhibit an immunostimulatory response, which in certain cases may be undesirable. For example, Galbraith et al. (*Antisense Res. & Dev.* (1994) **4**:201-206) disclose complement activation by some PS oligonucleotides. Henry et al. (*Pharm. Res.* (1994) **11**: PPDM8082) disclose that some PS oligonucleotides may potentially interfere with blood clotting.

There is, therefore, a need for modified oligonucleotides directed to cancer-related genes that retain gene expression inhibition properties while producing fewer side effects than conventional oligonucleotides.

SUMMARY OF THE INVENTION

The present invention relates to modified oligonucleotides useful for studies of gene expression and for the antisense therapeutic approach. The invention provides modified oligonucleotides that down-regulate the expression of the RI_{α} gene while producing fewer side effects than conventional oligonucleotides. In particular, the invention provides modified oligonucleotides that demonstrate reduced mitogenicity, reduced activation of complement and reduced antithrombotic properties, relative to conventional oligonucleotides.

It is also known that some PS oligonucleotides cause an immunostimulatory response in subjects to whom they have been

administered, which may be undesirable in some cases.

It is known that exclusively phosphodiester- or exclusively phosphorothioate-linked oligonucleotides directed to the first 100 nucleotides of the RI_α nucleic acid inhibit cell proliferation.

It has now been discovered that modified oligonucleotides complementary to the protein kinase A RI_α subunit gene inhibit the growth of tumors *in vivo* with at least the activity of a comparable PO- or PS-linked oligonucleotide with fewer side effects.

It has now further been discovered that modified oligonucleotides complementary to the protein kinase A RI_α subunit gene have a synergistic growth inhibitory effect with antibodies that bind to epidermal growth factor receptor (EGFR) or with various classes of cytotoxic drugs, including taxanes, platinum-derived agents, and topoisomerase II-selective drugs.

These findings have been exploited to produce the present invention, which in a first aspect, includes synthetic hybrid, inverted hybrid, and inverted chimeric oligonucleotides and compositions of matter for specifically down-regulating protein kinase A subunit RI_α gene expression with reduced side effects. Such inhibition of gene expression is useful as an

alternative to mutant analysis for determining the biological function and role of protein kinase A-related genes in cell proliferation and tumor growth. Such inhibition of RI_{α} gene expression can also be used to therapeutically treat diseases and disorders that are caused by the over-expression or inappropriate expression of the gene.

As used herein, the term "synthetic oligonucleotide" includes chemically synthesized polymers of three up to 50, preferably from about 15 to about 30, and most preferably, 18 ribonucleotide and/or deoxyribonucleotide monomers connected together or linked by at least one, and preferably more than one, 5' to 3' internucleotide linkage.

For purposes of the invention, the terms "oligonucleotide sequence that is complementary to a genomic region or an RNA molecule transcribed therefrom" and "oligonucleotide complementary to" are intended to mean an oligonucleotide that binds to the target nucleic acid sequence under physiological conditions, e.g., by Watson-Crick base pairing (interaction between oligonucleotide and single-stranded nucleic acid) or by Hoogsteen base pairing (interaction between oligonucleotide and double-stranded nucleic acid) or by any other means including in the case of a oligonucleotide binding to RNA, causing pseudoknot formation. Binding by Watson-Crick or Hoogsteen base pairing under physiological conditions is measured as a

practical matter by observing interference with the function of the nucleic acid sequence.

In one preferred embodiment according to this aspect of the invention, the oligonucleotide is a core region hybrid oligonucleotide comprising a region of at least two deoxyribonucleotides, flanked by 5′ and 3′ ribonucleotide regions, each having at least four ribonucleotides. A hybrid oligonucleotide having the sequence set forth in the Sequence Listing as SEQ ID NO:4 is one particular embodiment. In some embodiments, each of the 3′ and 5′ flanking ribonucleotide regions of an oligonucleotide of the invention comprises at least four contiguous, 2′-O-substituted ribonucleotides.

For purposes of the invention, the term "2′-O-substituted" means substitution of the 2′ position of the pentose moiety with an -O- lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an -O-aryl or allyl group having 2-6 carbon atoms, wherein such alkyl, aryl or allyl group may be unsubstituted or may be substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl, or amino groups; or with a hydroxy, an amino or a halo group, but not with a 2′-H group.

In some embodiments, each of the 3′ and 5′ flanking ribonucleotide regions of an oligonucleotide of the invention comprises at least one 2′-O-alkyl substituted ribonucleotide.

In one preferred embodiment, the 2′-O-alkyl-substituted nucleotide is a 2′-O-methyl ribonucleotide. In other preferred embodiments, the 3′ and 5′ flanking ribonucleotide regions of an oligonucleotide of the invention comprises at least four 2′-O-methyl ribonucleotides. In preferred embodiments, the ribonucleotides and deoxyribonucleotides of the hybrid oligonucleotide are linked by phosphorothioate internucleotide linkages. In particular embodiments, this phosphorothioate region or regions have from about four to about 18 nucleosides joined to each other by 5′ to 3′ phosphorothioate linkages, and preferably from about 5 to about 18 such phosphorothioate-linked nucleosides. The phosphorothioate linkages may be mixed R_p and S_p enantiomers, or they may be stereoregular or substantially stereoregular in either R_p or S_p form (see Iyer et al. (1995) *Tetrahedron Asymmetry* **6:**1051-1054).

In another preferred embodiment according to this aspect of the invention, the oligonucleotide is an inverted hybrid oligonucleotide comprising a region of at least four ribonucleotides flanked by 3′ and 5′ deoxyribonucleotide regions of at least two deoxyribonucleotides. The structure of this oligonucleotide is "inverted" relative to traditional hybrid oligonucleotides. In some embodiments, the 2′-O-substituted RNA region has from about four to about ten 2′-O-substituted nucleosides joined to each other by 5′ to 3′ internucleoside linkages, and most preferably from about four to about six such 2′-O-substituted

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nucleosides. In some embodiments, the oligonucleotides of the invention have a ribonucleotide region comprises at least five contiguous ribonucleotides. In one particularly preferred embodiment, the overall size of the inverted hybrid oligonucleotide is 18. In preferred embodiments, the 2'-O-substituted ribonucleosides are linked to each other through a 5' to 3' phosphorothioate, phosphorodithioate, phosphotriester, or phosphodiester linkages. The phosphorothioate 3' or 5' flanking region (or regions) has from about four to about 18 nucleosides joined to each other by 5' to 3' phosphorothioate linkages, and preferably from about 5 to about 18 such phosphorothioate-linked nucleosides. In preferred embodiments, the phosphorothioate regions will have at least 5 phosphorothioate-linked nucleosides. One specific embodiment is an oligonucleotide having substantially the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:6. In preferred embodiments of this aspect of the invention, the ribonucleotide region comprise 2'-O-substituted ribonucleotides, such as 2'-O-alkyl substituted ribonucleotides. One particularly preferred embodiment is a hybrid oligonucleotide whose ribonucleotide region comprise at least one 2'-O-methyl ribonucleotide.

SUB BS CONT

In some embodiments, all of the nucleotides in the inverted hybrid oligonucleotide are linked by phosphorothioate internucleotide linkages. In particular embodiments, the deoxyribonucleotide flanking region or regions has from about four to

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about 18 nucleosides joined to each other by 5′ to 3′ phosphorothioate linkages, and preferably from about 5 to about 18 such phosphorothioate-linked nucleosides. In some embodiments, the deoxyribonucleotide 3′ and 5′ flanking regions of the hybrid oligonucleotides of the invention have about 5 phosphorothioate-linked nucleosides. The phosphorothioate linkages may be mixed R_p and S_p enantiomers, or they may be stereoregular or substantially stereoregular in either R_p or S_p form (see Iyer et al. (1995) *Tetrahedron Asymmetry* **6:**1051-1054).

Another embodiment is a composition of matter for inhibiting the expression of protein kinase A subunit RI_α with reduced side effects, the composition comprising an inverted hybrid oligonucleotide according to the invention.

Yet another preferred embodiment according to this aspect of the invention is an inverted chimeric oligonucleotide comprising an oligonucleotide nonionic region of at least four nucleotides flanked by one or more, and preferably two oligonucleotide phosphorothioate regions. Such a chimeric oligonucleotide has a structure that is "inverted" relative to traditional chimeric oligonucleotides. In one particular embodiment, an inverted chimeric oligonucleotide of the invention has substantially the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:1. In preferred embodiments, the oligonucleotide nonionic region comprises about four to about 12 nucleotides joined to each other

by 5′ to 3′ nonionic linkages. In some embodiments, the nonionic region contains alkylphosphonate and/or phosphoramidate and/or phosphotriester internucleoside linkages. In one particular embodiment, the oligonucleotide nonionic region comprises six nucleotides. In some preferred embodiments, the oligonucleotide has a nonionic region having from about six to about eight methylphosphonate-linked nucleosides, flanked on either side by phosphorothioate regions, each having from about six to about ten phosphorothioate-linked nucleosides. In preferred embodiments, the flanking region or regions are phosphorothioate nucleotides. In some embodiments, the flanking region or regions have from about four to about 24 nucleosides joined to each other by 5′ to 3′ phosphorothioate linkages, and preferably from about six to about 16 such phosphorothioate-linked nucleosides. In preferred embodiments, the phosphorothioate regions have from about five to about 15 phosphorothioate-linked nucleosides. The phosphorothioate linkages may be mixed R_p and S_p enantiomers, or they may be stereoregular or substantially stereoregular in either R_p or S_p form (see Iyer et al. (1995) *Tetrahedron Asymmetry* **6**:1051-1054).

Another embodiment of this aspect of the invention is a composition of matter for inhibiting the expression of protein kinase A subunit RI_α with reduced side effects, the composition comprising an inverted chimeric oligonucleotide according to the invention.

Another aspect of the invention is a method of inhibiting the proliferation of cancer cells *in vitro*. In this method, an oligonucleotide of the invention is administered to the cells.

Yet another aspect is a therapeutic composition comprising an oligonucleotide of the invention in a pharmaceutically acceptable carrier.

A method of treating cancer in an afflicted subject with reduced side effects is another aspect of the invention. This method comprises administering a therapeutic composition of the invention to the subject in which the protein kinase A subunit RI_{α} gene is being over-expressed.

In yet another aspect, the invention provides a method for inhibiting proliferation of cancer cells comprising:

(a) administering to the cells a first agent comprising a synthetic, modified oligonucleotide complementary to, and capable of down-regulating the expression of, nucleic acid encoding protein kinase A subunit RIα, the modified oligonucleotide having from about 15 to about 30 nucleotides and being a hybrid, inverted hybrid, or inverted chimeric oligonucleotide,

the hybrid oligonucleotide comprising a region of at least two deoxyribonucleotides, flanked by 3′ and 5′ flanking ribonucleotide regions each having at least four ribonucleotides,

the inverted hybrid oligonucleotide comprising a region of at least four

ribonucleotides flanked by 3′ and 5′ flanking deoxyribonucleotide regions of at least two deoxyribonucleotides,

and the inverted chimeric oligonucleotide comprising an oligonucleotide nonionic region of at least four nucleotides flanked by two oligonucleotide phosphorothioate regions; and

(b) administering to the cells a second agent comprising an antibody that binds to epidermal growth factor receptor (EGFR) or a cytotoxic agent selected from the group consisting of taxanes, platinum-derived agents, and topoisomeraseII-selective drugs;

wherein the administering steps may be performed simultaneously or sequentially in any order.

In yet another aspect, the invention provides a pharmaceutical composition comprising:

(a) a first agent comprising a synthetic, modified oligonucleotide complementary to, and capable of down-regulating the expression of, nucleic acid encoding protein kinase A subunit RIα, the modified oligonucleotide having from about 15 to about 30 nucleotides and being a hybrid, inverted hybrid, or inverted chimeric oligonucleotide,

the hybrid oligonucleotide comprising a region of at least two deoxyribonucleotides, flanked by 3′ and 5′ flanking ribonucleotide regions each having at least four ribonucleotides,

the inverted hybrid oligonucleotide comprising a region of at least four ribonucleotides flanked by 3′ and 5′ flanking

deoxyribonucleotide regions of at least two deoxyribonucleotides,

and the inverted chimeric oligonucleotide comprising an oligonucleotide nonionic region of at least four nucleotides flanked by two oligonucleotide phosphorothioate regions; and

(b) a second agent comprising an antibody that binds to epidermal growth factor receptor (EGFR) or a cytotoxic agent selected from the group consisting of taxanes, platinum-derived agents, and topoisomeraseII-selective drugs.

In still yet another aspect, the invention provides a method for treating cancer in an afflicted subject comprising:

(a) administering to the cells a first agent comprising a synthetic, modified oligonucleotide complementary to, and capable of down-regulating the expression of, nucleic acid encoding protein kinase A subunit RIα, the modified oligonucleotide having from about 15 to about 30 nucleotides and being a hybrid, inverted hybrid, or inverted chimeric oligonucleotide,

the hybrid oligonucleotide comprising a region of at least two deoxyribonucleotides, flanked by 3′ and 5′ flanking ribonucleotide regions each having at least four ribonucleotides,

the inverted hybrid oligonucleotide comprising a region of at least four ribonucleotides flanked by 3′ and 5′ flanking deoxyribonucleotide regions of at least two deoxyribonucleotides,

and the inverted chimeric oligonucleotide comprising an oligonucleotide nonionic region of

at least four nucleotides flanked by two oligonucleotide phosphorothioate regions; and

(b) administering to the cells a second agent comprising an antibody that binds to epidermal growth factor receptor (EGFR) or a cytotoxic agent selected from the group consisting of taxanes, platinum-derived agents, and topoisomeraseII-selective drugs;

wherein the administering steps may be performed simultaneously or sequentially in any order.

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Those skilled in the art will recognize that the elements of these preferred embodiments can be combined and the inventor does contemplate such combination. For example, 2'-O-substituted ribonucleotide regions may well include from one to all nonionic internucleoside linkages. Alternatively, nonionic regions may have from one to all 2'-O-substituted ribonucleotides. Moreover, oligonucleotides according to the invention may contain combinations of one or more 2'-O-substituted ribonucleotide region and one or more nonionic region, either or both being flanked by phosphorothioate regions. (See *Nucleosides & Nucleotides* **14:**1031-1035 (1995) for relevant synthetic techniques.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

FIG. 1 is a graphic representation showing the effect of modified oligonucleotides of the invention on tumor size in a mouse relative to various controls.

FIG. 2 is a graphic representation showing the effect of HYB 165 with docetaxel and monoclonal antibody MAb C225 on the growth of ZR75-1 human breast cancer cells.

FIG. 3 is a graphic representation showing the effect of HYB 508 with docetaxel and monoclonal anitbody MAb C225 on the growth of ZR75-1 human breast cancer cells.

FIG. 4 is a graphic representation showing the effect of HYB 165 with or without paclitaxel on the growth of geo human colon cancer cells.

FIG. 5 is a graphic representation showing the effect of HYB 165 and its control HYB 508 on the growth of 1A9PTX22 human ovarian cancer cells.

FIG. 6 is a graphic representation showing the effect of HYB 165 and its control HYB 508 on the growth of 1A9PTX10 human ovarian cancer cells.

FIG. 7 is a graphic representation showing the effect of HYB 165 and its control HYB 508 on the growth of 1A9 human ovarian cancer cells.

FIG. 8 is a graphic representation showing the effect of HYB 508 with or without monoclonal antibody MAb C225 on the growth of ZR75-1 human breast cancer cells.

FIG. 9 is a graphic representation showing the effect of HYB 165 and HYB 618 on the growth of OVCAR-3 ovarian cancer cells.

FIG. 10 is a graphic representation showing the effect of HYB 165 with or without docetaxel on the growth of ZR75-1 human breast cancer cells.

FIG. 11 is a graphic representation showing the effect of HYB 508 with or without docetaxel on the growth of ZR75-1 human breast cancer cells.

FIG. 12 is a graphic representation showing the effect of HYB 165 with or without monoclonal antibody MAb C225 on the growth of ZR75-1 human breast cancer cells.

FIG. 13 is a graphic representation showing the effect of HYB 165 and HYB 295 on the growth of ZR75-1 human breast cancer cells.

FIG. 14 is a graphic representation showing the effect of HYB 165 and HYB 508 on the growth of ZR75-1 human breast cancer cells.

FIG. 15 is a graphic representation showing the effect of HYB 165 and HYB 295 on the growth of geo colon cancer cells.

FIG. 16A is a graphic representation of data showing that the hybrid MBO antisense RI∝ inhibits tumor growth after i.p. administration.

FIG. 16B is a graphic representation of data showing that the hybrid MBO antisense RI∝ inhibits tumor growth after oral administration.

FIG. 17A is a graphic representation of data showing that oral hybrid MBO antisense RI∝ cooperatively inhibits tumor growth with taxol.

FIG. 17B is a graphic representation of data showing that oral hybrid MBO antisense RI∝ cooperatively increases survival in combination with taxol.

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FIG. 18 is a tabular representation of histochemical analysis of GEO tumors following treatment with taxol and/or different oral MBOs. .

DESCRIPTION OF THE PREFERRED EMBODIMENT

The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. The issued U.S. patents, allowed applications, published foreign applications, and references cited herein are hereby incorporated by reference.

Synthetic oligonucleotides of the hybrid, inverted hybrid, and inverted chimeric oligonucleotides as described above.

Such synthetic hybrid, inverted hybrid, and inverted chimeric oligonucleotides of the invention have a nucleotide sequence complementary to a genomic region or an RNA molecule transcribed therefore encoding the RI_{α} subunit of PKA. These oligonucleotides are about 15 to about 30 nucleotides in length, preferably about 15 to 25 nucleotides in length, but most preferably, are about 18 nucleotides long. The sequence of this gene is known. Thus, an oligonucleotide of the invention can have any nucleotide sequence complementary to any region of the gene. Three non-limiting examples of an 18mer of the invention has the sequence set forth below in TABLE 1 as SEQ ID NOS:1, 4, and 6.

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TABLE 1

Oligo #	Sequence (5′ → 3′)	Type	SEQ ID NO:
164	GCG TGC CTC CTC ACT GGC	Control	1
167	GCG **C**GC CTC CTC **G**CT GGC	Mismatched Control	2
188	GC**A** TGC **T**TC C**A**C AC**A** GGC	Mismatched Control	3
165	*** * * *** GCG UGC CTC CTC ACU GGC	Hybrid	4
168	*** * *. *** GCG **C**GC CTC CTC **G**CU GGC	Mismatched Hybrid (Control)	5
166	*** ** GCG TGC CUC CUC ACT GGC	Inverted Hybrid	6
169	*** ** GCG **C**GC CUC CUC **G**CT GGC	Mismatched Inverted Hybrid (Control)	7
189	*** ** GC**A** TGC **A**UC C**G**C AC**A** GGC	Mismatched Inverted Hybrid (Control)	8
190	••• ••• GCG TGC CTC CTC ACT GGC	Inverted Chimeric	1
191	••• ••• GCG **C**GC CTC CTC **G**CT GGC	Mismatched Inverted Chimeric (Control)	2

X = mismatched bases

* ribonucleotide

• methylphosphonate nucleotide

Oligonucleotides having greater than 18 oligonucleotides are also contemplated by the invention. These oligonucleotides have up to 25 additional nucleotides extending from the 3′, or 5′ terminus, or from both the 3′ and 5′ termini of, for example, the 18mer with SEQ ID NOS:1, 4,

or 6, without diminishing the ability of these oligonucleotides to down regulate RI_α gene expression. Alternatively, other oligonucleotides of the invention may have fewer nucleotides than, for example, oligonucleotides having SEQ ID NOS:1, 4, or 6. Such shortened oligonucleotides maintain at least the antisense activity of the parent oligonucleotide to down-regulate the expression of the RI_α gene, or have greater activity.

The oligonucleotides of the invention can be prepared by art recognized methods. Oligonucleotides with phosphorothioate linkages can be prepared manually or by an automated synthesizer and then processed using methods well known in the field such as phosphoramidite (reviewed in Agrawal et al. (1992) *Trends Biotechnol.* **10**:152-158, *see, e.g.*, Agrawal et al. (1988) *Proc. Natl. Acad. Sci.* (USA) **85**:7079-7083) or H-phosphonate (*see, e.g.*, Froehler (1986) *Tetrahedron Lett.* **27**:5575-5578) chemistry. The synthetic methods described in Bergot et al. (*J. Chromatog.* (1992) **559**:35-42) can also be used. Examples of other chemical groups include alkylphosphonates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, 2'-O-methyls, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters. Oligonucleotides with these linkages can be prepared according to known methods (*see, e.g.*, Goodchild (1990) *Bioconjugate Chem.* **2:**165-187; Agrawal et al. (*Proc. Natl. Acad. Sci.* (USA) (1988) **85**:7079-7083); Uhlmann et al. (*Chem. Rev.* (1990) **90**:534-583;

and Agrawal et al. (*Trends Biotechnol.* (1992) **10**:152-158)).

Preferred hybrid, inverted hybrid, and inverted chimeric oligonucleotides of the invention may have other modifications which do not substantially affect their ability to specifically down-regulate RI_α gene expression. These modifications include those which are internal or are at the end(s) of the oligonucleotide molecule and include additions to the molecule at the internucleoside phosphate linkages, such as cholesteryl or diamine compounds with varying numbers of carbon residues between the two amino groups, and terminal ribose, deoxyribose and phosphate modifications which cleave, or crosslink to the opposite chains or to associated enzymes or other proteins which bind to the RI_α nucleic acid. Examples of such oligonucleotides include those with a modified base and/or sugar such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide having a sugar which, at one or both its 3' and 5' positions is attached to a chemical group other than a hydroxyl or phosphate group (at its 3' or 5' position). Other modified oligonucleotides are capped with a nuclease resistance-conferring bulky substituent at their 3' and/or 5' end(s), or have a substitution in one or both nonbridging oxygens per nucleotide. Such modifications can be at some or all of the internucleoside linkages, as well as at either or both ends of the oligonucleotide and/or in the interior of the molecule (reviewed

in Agrawal et al. (1992) *Trends Biotechnol.* **10**:152-158).

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The invention also provides therapeutic compositions suitable for treating undesirable, uncontrolled cell proliferation or cancer comprise at least one oligonucleotide in accordance with the invention, capable of specifically down-regulating expression of the RI_{α} gene, and a pharmaceutically acceptable carrier or diluent. It is preferred that an oligonucleotide used in the therapeutic composition of the invention be complementary to at least a portion of the RI_{α} genomic region, gene, or RNA transcript thereof.

SUB B11

As used herein, a "pharmaceutically or physiologically acceptable carrier" includes any and all solvents (including but limited to lactose), dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions of the invention is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

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Several preferred therapeutic composition of the invention suitable for inhibiting cell proliferation *in vitro* or *in vivo* or for treating cancer in humans in accordance with the methods of

the invention comprises about 25 to 75 mg of a lyophilized oligonucleotide(s) having SEQ ID NOS:1, 4, and/or 6 and 20-75 mg lactose, USP, which is reconstituted with sterile normal saline to the therapeutically effective dosages described herein.

SUB B12 CONT

In another aspect, the invention provides pharmaceutical compositions comprising a modified oligonucleotide of the invention in combination with an antibody that binds to epidermal growth factor receptor (EGFR) or a cytotoxic agent. Preferred cytotoxic agents include, without limitation, taxanes, platinum-derived agents, and topoisomeras II-selective drugs.

SUB B13

The invention also provides methods for treating humans suffering from disorders or diseases wherein the RI_{α} gene is incorrectly or over-expressed. Such a disorder or disease that could be treated using this method includes tumor-forming cancers such as, but not limited to, human colon carcinoma, breast carcinoma, gastric carcinoma, and neuroblastoma. In the method of the invention, a therapeutically effective amount of a composition of the invention is administered to the human. Such methods of treatment according to the invention, may be administered in conjunction with other therapeutic agents.

In certain preferred embodiments, the methods of treatment according to the invention comprise a) administering a first agent comprising a synthetic, modified oligonucleotide complementary

to, and capable of down-regulating the expression of, nucleic acid encoding protein kinase A subunit RIα according to the invention; and b) administering a second agent comprising an antibody that binds to epidermal growth factor receptor (EGFR) or a cytotoxic agent selected from the group consisting of taxanes, platinum-derived agents, and topoisomeraseII-selective drugs. In some preferred embodiments according to this aspect of the invention, the two agents are administered simultaneously. In certain preferred embodiments, the second agent is administered prior to administration of the first agent.

In certain preferred embodiments, the second agent is a taxane, including but not limited to paclitaxel and docetaxel. Preferably, paclitaxel is administered in doses of up to 300 mg/m^2/dose by intravenous infusion (1 hour to 24 hour duration), given at a frequency of every 21 days or less. Preferably, docetaxel is administered in doses of up to 300 mg/m^2/dose by intravenous infusion (1 hour to 24 hour duration), given at a frequency of every 21 days or less.

In certain other preferred embodiments, the second agent is an antibody that binds to epidermal growth factor receptor. Preferably, the antibody is a monoclonal antibody, more preferably a humanized monoclonal antibody. In certain preferred embodiments, the monoclonal antibody is C225 (N.I. Goldstein *et al.*, *Clin. Cancer Res.*, 1(11):1311-8 (1995). Preferably, C225 is administered in doses of up to 500 mg/m^2/dose by

intravenous infusion (10 minutes to 24 hour duration), given at a frequency of every 28 days or less.

SUB B14

In preferred embodiments according to this aspect of the invention, the first agent is a synthetic modified oligonucletotide having a sequence oligonucleotide has a nucleotide sequence consisting essentially of the nucleotide sequence set forth in SEQ ID NO:4. Preferably, the oligonucleotide is administer at a dose of up ot 540 mg/m^2/dose by intravenous infusion (2 hours to 21 days in duration or up to 1,050 mg/m^2/day by oral or rectal administration.

As used herein, the term "therapeutically effective amount" means the total amount of each active component of the pharmaceutical formulation or method that is sufficient to show a meaningful subject or patient benefit, i.e., a reduction in tumor growth or in the expression of proteins which cause or characterize the cancer. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

SUB B15

A "therapeutically effective manner" refers to a route, duration, and frequency of administration of the pharmaceutical formulation which ultimately results in meaningful patient

benefit, as described above. In some embodiments of the invention, the pharmaceutical formulation is administered via injection, sublingually, rectally, intradermally, orally, or enterally in bolus, continuous, intermittent, or continuous, followed by intermittent regimens.

The therapeutically effective amount of synthetic oligonucleotide in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patent has undergone. Ultimately, the attending physician will decide the amount of synthetic oligonucleotide with which to treat each individual patient. Initially, the attending physician will administer low doses of the synthetic oligonucleotide and observe the patient's response. Larger doses of synthetic oligonucleotide may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further. It is contemplated that the dosages of the pharmaceutical compositions administered in the method of the present invention should contain about 0.1 to 5.0 mg/kg body weight per day, and preferably 0.1 to 2.0 mg/kg body weight per day. When administered systemically, the therapeutic composition is preferably administered at a sufficient dosage to attain a blood level of oligonucleotide from about 0.01 μM to about 10 μM. Preferably, the concentration of oligonucleotide at the site of aberrant gene expression should be from about 0.01

μM to about 10 μM, and most preferably from about 0.05 μM to about 5 μM. However, for localized administration, much lower concentrations than this may be effective, and much higher concentrations may be tolerated. It may be desirable to administer simultaneously or sequentially a therapeutically effective amount of one or more of the therapeutic compositions of the invention when individual as a single treatment episode.

Administration of pharmaceutical compositions in accordance with invention or to practice the method of the present invention can be carried out in a variety of conventional ways, such as by oral ingestion, enteral, rectal, or transdermal administration, inhalation, sublingual administration, or cutaneous, subcutaneous, intramuscular, intraocular, intraperitoneal, or intravenous injection, or any other route of administration known in the art for administrating therapeutic agents.

When the composition is to be administered orally, sublingually, or by any non-injectable route, the therapeutic formulation will preferably include a physiologically acceptable carrier, such as an inert diluent or an assimilable edible carrier with which the composition is administered. Suitable formulations that include pharmaceutically acceptable excipients for introducing compounds to the bloodstream by other than injection routes can be found in *Remington's Pharmaceutical Sciences* (18th ed.) (Genarro, ed. (1990)

Mack Publishing Co., Easton, PA). The oligonucleotide and other ingredients may be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the individual's diet. The therapeutic compositions may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. When the therapeutic composition is administered orally, it may be mixed with other food forms and pharmaceutically acceptable flavor enhancers. When the therapeutic composition is administered enterally, they may be introduced in a solid, semi-solid, suspension, or emulsion form and may be compounded with any number of well-known, pharmaceutically acceptable additives. Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are also contemplated such as those described in U.S. Patent Nos. 4,704,295, 4,556,552, 4,309,404, and 4,309,406.

When a therapeutically effective amount of composition of the invention is administered by injection, the synthetic oligonucleotide will preferably be in the form of a pyrogen-free, parenterally-acceptable, aqueous solution. The preparation of such parenterally-acceptable solutions, having due regard to ph, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for injection should contain, in addition to the synthetic oligonucleotide, an isotonic vehicle

such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile. It must be stable under the conditions of manufacture and storage and may be preserved against the contaminating action of microorganisms, such as bacterial and fungi. The carrier can be a solvent or dispersion medium. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents. Prolonged absorption of the injectable therapeutic agents can be brought about by the use of the compositions of agents delaying absorption. Sterile injectable solutions are prepared by incorporating the oligonucleotide in the required amount in the appropriate solvent, followed by filtered sterilization.

SUB B18

The pharmaceutical formulation can be administered in bolus, continuous, or intermittent dosages, or in a combination of continuous and intermittent dosages, as determined by the physician and the degree and/or stage of illness

of the patient. The duration of therapy using the pharmaceutical composition of the present invention will vary, depending on the unique characteristics of the oligonucleotide and the particular therapeutic effect to be achieved, the limitations inherent in the art of preparing such a therapeutic formulation for the treatment of humans, the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

Compositions of the invention are useful for inhibiting or reducing the proliferation of cancer or tumor cells *in vitro*. A synthetic oligonucleotide of the invention is administered to the cells in an amount sufficient to enable the binding of the oligonucleotide to a complementary genomic region or RNA molecule transcribed therefrom encoding the RI_{α} subunit. In this way, expression of PKA is decreased, thus inhibiting or reducing cell proliferation.

Compositions of the invention are also useful for treating cancer or uncontrolled cell proliferation in humans. In this method, a therapeutic formulation including an antisense oligonucleotide of the invention is provided in a physiologically acceptable carrier. The individual is then treated with the therapeutic formulation in an amount sufficient to enable the

binding of the oligonucleotide to the PKA RI_α genomic region or RNA molecule transcribed therefrom in the infected cells. In this way, the binding of the oligonucleotide inhibits or down-regulates RI_α expression and hence the activity of PKA.

In practicing the method of treatment or use of the present invention, a therapeutically effective amount of at least one or more therapeutic compositions of the invention is administered to a subject afflicted with a cancer. An anticancer response showing a decrease in tumor growth or size or a decrease in RI_α expression is considered to be a positive indication of the ability of the method and pharmaceutical formulation to inhibit or reduce cell growth and thus, to treat cancer in humans.

SUB B19

At least one therapeutic composition of the invention may be administered in accordance with the method of the invention either alone or in combination with other known therapies for cancer such as cisplatin, carboplatin, paclitaxol, tamoxifen, taxol, interferon α and doxorubicin. When co-administered with one or more other therapies, the compositions of the invention may be administered either simultaneously with the other treatment(s), or sequentially. If administered sequentially, the attending physician will decide on the appropriate sequence of administering the compositions of the invention in combination with the other therapy.

The following examples illustrate the preferred modes of making and practicing the present invention, but are not meant to limit the scope of the invention since alternative methods may be utilized to obtain similar results.

EXAMPLE 1

Synthesis, Deprotection, and Purification of Oligonucleotides

Oligonucleotides were synthesized using standard phosphoramidite chemistry (Beaucage (1993) *Meth. Mol. Biol.* **20**:33-61) on an automated DNA synthesizer (Model 8700, Biosearch, Bedford, MA) using a beta-cyanoethyl phosphoramidate approach.

Oligonucleotide phosphorothioates were synthesized using an automated DNA synthesizer (Model 8700, Biosearch, Bedford, MA) using a beta-cyanoethyl phosphoramidate approach on a 10 micromole scale. To generate the phosphorothioate linkages, the intermediate phosphite linkage obtained after each coupling was oxidized using 3H, 1,2-benzodithiole-3H-one-1,1-dioxide (see Beaucage, in *Protocols for Oligonucleotides and Analogs: Synthesis and Properties*, Agrawal (ed.), (1993) Humana Press, Totowa, NJ, pp. 33-62). Similar synthesis was carried out to generate phosphodiester linkages, except that a standard oxidation was carried out using standard iodine reagent. Synthesis of inverted chimeric oligonucleotide was carried out in the same manner, except that methylphosphonate linkages were assembled using nucleoside methylphosphonamidite (Glen Research, Sterling,

VA), followed by oxidation with 0.1 M iodine in tetrahydrofuran/2,6-lutidine/water (75:25:0.25) (see Agrawal & Goodchild (1987) *Tet. Lett.* **28**:3539-3542). Hybrids and inverted hybrid oligonucleotides were synthesized similarly, except that the segment containing 2'-O-methylribonucleotides was assembled using 2'-O-methylribonucleoside phosphoramidite, followed by oxidation to a phosphorothioate or phosphodiester linkage as described above. Deprotection and purification of oligonucleotides was carried out according to standard procedures, (see Padmapriya et al. (1994) *Antisense Res. & Dev.* **4**:185-199), except for oligonucleotides containing methylphosphonate-containing regions. For those oligonucleotides, the CPG-bound oligonucleotide was treated with concentrated ammonium hydroxide for 1 hour at room temperature, and the supernatant was removed and evaporated to obtain a pale yellow residue, which was then treated with a mixture of ethylenediamine/ethanol (1:1 v/v) for 6 hours at room temperature and dried again under reduced pressure.

EXAMPLE 2

Propagation and Quantitation of Cell Lines and Virus Stocks

The cell line utilized was the CEM-SS cell line (Southern Research Institute-Frederick Research Center, Frederick, MD). These cells are highly susceptible to infection with HIV, rapidly form multinucleated syncytia, and are eventually killed by HIV. The cells were maintained (2-7 x

10^5 cells per ml) in RPMI 1640 tissue culture medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics, and were passaged twice weekly at 1:20 dilution. Passage number was logged each week. Cells were discarded after twenty weeks of passage and fresh CEM-SS cells thawed and utilized in the assay. Stocks of CEM-SS cells were frozen in liquid nitrogen in 1 ml NUNC vials in 90% fetal calf serum and 10% dimethyl sulfoxide (DMSO). Following thawing, CEM-SS cells were routinely ready to be utilized in the primary screen assay after two weeks in culture. Prior to replacing a late passage cell line, the new CEM-SS cells weree tested in the screening assay protocol utilizing the current stock of infectious virus and AZT. If the infectivity of the virus was significantly different on the new cells or if AZT appeared less active than expected the new cells were not entered into the screening program. Mycoplasma testing was routinely performed on all cell lines.

Virus utilized Southern Research Institute-Frederick Research Center. Virus pools were prepared and titrated in CEM-SS cells, placed in 5 ml aliquots, and frozen at -135°C. After thawing, unused virus is discarded to avoid changes in infectious titer. Virus pools were prepared by the acute infection of 5 x 10^5 CEM-SS cells with HIV in a volume of 200 μl at a multiplicity of infection determined to give complete cell killing at day 7 post-infection (approximately 0.05 for the III_B isolate of HIV-1 and 0.01 for the RF isolate of HIV-1). Infection was allowed to

proceed for one hour at 37°C, after which the cells were transferred to a T25 flask and the volume increased to 2 ml. On day 1 post-infection the volume was brought to 5 ml and on day 2 the volume was increased to 10 ml. Beginning on day 4, the cells were pelleted, the supernatant saved, and the cells resuspended in a fresh 10 ml aliquot of tissue culture medium. Complete medium changes on a daily basis, rather than allowing growth of the cells in the medium for longer periods of time, allowed the virus inoculum utilized in the primary screen to remain relatively undepleted of nutrients when it is used to infect cells. The staining reaction utilized (XTT, see method below) required that the glucose concentration remain high (161). Wells depleted of glucose by cell growth will not permit metabolic conversion of the tetrazolium dye to the formazan product.

Cell-free supernatants from the acutely infected cells were saved on day 4, day 5, day 6, and day 7. An aliquot of supernatant was saved separately on each day for use in titer determination. Titer determinations included reverse transcriptase activity assay (see below), endpoint titration or plaque assay (CEM-SS) quantification of infectious particles (see below), and quantification of cell killing kinetics.

It has been determined that peak levels of infectious virus are produced in the acutely infected cultures as the viability of the cells falls through the 50% level. Since the primary

screening assay quantifies the protective effects of a compound by its ability to inhibit HIV-induced cytopathic effects, the quantity of virus required to kill CEM-SS cells in 6 days was routinely utilized to determine the amount of virus required per well in the primary screening assay. Each of the daily pools was titrated in the primary screening tetrazolium dye XTT assay protocol by performing two-fold dilutions of the virus beginning at a high test concentration of 50 µl of virus per well. The XTT staining method was utilized to determine the exact amount of virus required to kill all of the CEM-SS cells in each well and this minimum amount of virus was utilized for performance of all primary testing. Identical methods were utilized to prepare all virus isolates utilized, including laboratory-derived strains of HIV-1, HIV-2 and SIV. Clinical isolates utilized were passaged in fresh human cells. The methods for the growth of these cells and the production of virus pools is described below.

SUB B2

Titer determinations

reverse transcriptase activity assay (see methods below), endpoint titration or plaque assay (CEM-SS) quantification of infectious particles (see methods below), and quantification of cell killing kinetics.

Microtiter Antiviral XTT Assay

The tetrazolium dye XTT staining method was utilized to determine the exact amount of virus

required to kill all of the CEM-SS cells in each well and this minimum amount of virus was utilized for performance of all primary testing.

Cell Preparation:

CEM-SS cells (or other established human cell line used in these experiments) were passaged in T-150 flasks for use in the assay. On the day preceding the assay, the cells were split I:2 to assure they would be in an exponential growth phase at time of infection. On the day of assay the cells were washed twice with tissue culture medium and resuspended in fresh tissue culture medium. Total cell and viability counting was performed using a hemacytometer and trypan blue dye exclusion. Cell viability was greater than 95% for the cells to be utilized in the assay. The cells were pelleted and resuspended at 2.5 x 10^4 cells per ml in tissue culture medium. Cells were added to the drug-containing plates in a volume of 50 μl.

Virus Preparation:

A pretitered aliquot of virus was removed from the freezer -80°C) and allowed to thaw slowly to room temperature in a biological safety cabinet. The virus was resuspended and diluted into tissue culture medium such that the amount of virus added to each well in a volume of 50 μl will be the amount determined to give complete cell killing at 6 days post-infection. In general the virus pools produced with the IIIB isolate of HIV required the addition of 5 μl of virus per well. Pools of RF

virus were five to ten-fold more potent, requiring 0.5-1 μl per well. $TCID_{50}$ calculation by endpoint titration in CEM-SS cells indicated that the multiplicity of infection of these assays ranged from 0.005-2.5.

Plate Format:

Each plate contained cell control wells (cells only), virus control wells (cells plus virus), drug toxicity control wells (cells plus drug only), drug colorimetric control wells (drug only) as well as experimental wells (drug plus cells plus virus).

XTT Staining of Screening Plates:

After 6 days of incubation at 37°C in a 5% CO_2 incubator the test plates were analyzed by staining with the tetrazolium dye XTT. XTT-tetrazolium is metabolized by the mitochondrial enzymes of metabolically active cells to a soluble formazan product, allowing the rapid quantitative analysis of the inhibition of HIV-induced cell killing by anti-HIV test substances. On day 6 post-infection plates were removed from the incubator and observed. The use of round bottom microtiter plates allows rapid macroscopic analysis of the activity of a given test compound by the evaluation of pellet size. The results of the macroscopic observations were confirmed and enhanced by further microscopic analysis.

XTT solution was prepared daily as a stock of 1 mg/ml in PBS. Phenazine methosulfate (PMS) solution was prepared at 15 mg/ml in PBS and stored in the dark at -20°C. XTT/PMS stock was prepared immediately before use by diluting the PMS 1:100 into PBS and adding 40 μl per ml of XTT solution. Fifty microliters of XTT/PMS was added to each well of the plate and the plate was incubated for an additional 4 hours at 37°C. Adhesive plate sealers were used in place of the lids, the sealed plate was inverted several times to mix the soluble formazan product and the plate was read spectrophotometrically at 450 nm with a Molecular Devices Vmax plate reader. Using an in-house computer program %CPE Reduction, %Cell Viability, $IC_{25,\ 50\ \&\ 95}$, $TC_{25,50\ \&\ 95}$ and other indices were calculated and the graphic results summary was displayed.

b. Reverse Transcriptase Activity Assay:

A microtiter based reverse transcriptase (RT) reaction was utilized (Buckheit *et al* (1991) *AIDS Research and Human Retroviruses* 7:295-302). Tritiated thymidine triphosphate (NEN) (TTP) was resuspended in distilled H_2O at 5 Ci/ml. Poly rA and oligo dT were prepared as a stock solution which was kept at -20°C. The RT reaction buffer was prepared fresh on a daily basis and consists of 125 μl 1M EGTA, 125 μl dH_2O, 125 μl Triton X-100, 50 μl 1M Tris(pH 7.4), 50 μl 1M DTT, and 40 μl 1M $MgCl_2$. These three solutions were mixed together in a ratio of 1 parts distilled water. Ten microliters of this reaction mixture was

placed in a round bottom microtiter plate and 15 μl of virus containing supernatant was added and mixed. The plate was incubated at 37°C and incubated for 60 minutes. Following reaction, the reaction volume was spotted onto filter mats, washed 6 times for 5 minutes each in a 5% sodium phosphate buffer, 2 times for 1 minute each in distilled water, 2 times for 1 minute each in 70% ethanol, and then dried. The dried filter mat was placed in a plastic sample bag, Betaplate scintillation fluid was added and the bag was heat-sealed. Incorporated radioactivity was quantified utilizing a Wallac Microbeta scintillation counter.

c. <u>p24 ELISA</u>:

ELISA kits were purchased from Coulter. The assay is performed according to the manufacturer's recommendations. Prior to ELISA analysis we routinely performed the reverse transcriptase activity assays (described above) and used the values for incorporated radioactivity in the RT activity assay to determine the dilution of our samples requires for the ELISA. We have constructed standard curves so that the dilutions of virus to be used in the p24 ELISA can be accurately determined from the RT activity assay. Control curves are generated in each assay to accurately quantify the amount of capsid protein in each sample. Data was obtained by spectrophotometric analysis at 450 nm using a Molecular Devices Vmax plate reader. P24 concentrations were calculated from the optical

SUB B22

density values by use of the Molecular Devices software package Soft Max.

d. Infectious Particles:

Infectious virus particles were qualified utilizing the CEM-SS plaque assay as described by Nara, P.L. and Fischinger, P.J. (1988) *Nature* 332:469-470). Flat bottom 96-well microtiter plates (Costar) were coated with 50 μl of poly-L-lysine (Sigma) at 50 μg/ml for 2 hours at 37°C. The wells were then washed with PBS and 2.5×10^5 CEM-SS cells were placed in the microtiter well where they became fixed to the bottom of the plate. Enough cells were added to form a monolayer of CEM-SS cells in each well. Virus containing supernatant was added from each well of the XTT plate, including virus and cell controls and each serial dilution of the test substance. The number of syncytia were qualified in the flat-bottom 96-well microtiter plate with an Olympus CK2 inverted microscope at 4 days following infection. Each syncytium resulted from a single infectious HIV virion.

Anti-HIV Activity in Fresh Human Cells: Assay in Fresh Human T-Lymphocytes

Fresh human peripheral blood lymphocytes (PBL) are isolated from voluntary Red Cross donors, seronegative for HIV and HBV. Leukophoresed blood is diluted 1:1 with Dulbecco's phosphate buffered saline (PBS), layered over 14 mL of Ficoll-Hypaque

density gradient in a 50 mL centrifuge tube. Tubes are then centrifuged for 30 minutes at 600 X g. Banded PBLs are gently aspirated from the resulting interface and subsequently washed 2X with PBS by low speed centrifugation. After final wash, cells are enumerated by trypan blue exclusion and re-suspended at 1 x 10^7/mL in RPMI 1640 with 15% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 4 mg/mL PHA-P and allowed to incubate for 48 - 72 hours at 37°C. After incubation, PBLs are centrifuged and reset in RPMI 1640 with 15% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL gentamycin, and 20 U/mL recombinant human IL-2. PBLs are maintained in this medium at a concentration of 1-2 x 10^6/mL with bi-weekly medium changes, until use in assay protocol.

For the PBL assay, PHA-P stimulated cells from at least two normal donors are pooled, set in fresh medium at 2 x 10^6/mL and plated in the interior wells of a 96 well round bottom microplate at 50 μL/well. Test drug dilutions are prepared at a 2X concentration in microtiter tubes and 100 μL of each concentration is placed in appropriate wells in a standard format. Fifty microliters of a predetermined dilution of virus stock is placed in each test well. Wells with cells and virus alone are used for virus control. Separate plates are identically set without virus for drug cytotoxicity studies using an XTT assay system.

In the standard PBL assay (MOI: 0.2), the assay was ended on day 7 following collection of cell

free supernatant samples for reverse transcriptase activity assay. In the low MOI PBL assay (MOI: 0.02), supernatant samples were collected on day 6, day 11, and day 14 post-infection and analyzed for RT activity. Tritiated thymidine triphosphate (NEN) (TTP) was resuspended in distilled H_2O at 5 Ci/ml. Poly rA and oligo dT were prepared as a stock solution which was kept at -20°C. The RT reaction buffer was prepared fresh on a daily basis and consists of 125 μl 1M DTT, and 40 μl 1M $MgCl_2$. These three solutions were mixed together in a ratio of 2 parts TTP, 1 part poly rA:oligo dT, and 1 part reaction buffer. Ten microliters of this reaction mixture was placed in a round bottom microtiter plate and 15 μl of virus containing supernatant was added and mixed. The plate was incubated at 37°C in a water bath with a solid support to prevent submersion of the plate and incubated for 60 minutes. Following reaction, the reaction volume was spotted onto pieces of DE81 paper, washed 5 times for 5 minutes each in a 5% sodium phosphate buffer, 2 times for 1 minute each in distilled water, 2 times for 1 minute each in 70% ethanol, and then dried. Opti-Fluor O was added to each sample and incorporated radioactivity was quantified utilizing a Wallac 1450 Microbetaplus liquid scintillation counter.

Tritiated thymidine incorporation was measured in parallel cultures at day 7. Each well was pulsed with 1 μCi of tritiated thymidine and the cells were harvested 18 hours later with a Skatron cell harvester onto glass fiber filter papers. The filters were dried, placed in a scintillation vial

with 1 ml of scintillation cocktail and incorporated radioactivity was quantified on a Packard Tri-Carb 1900 TR liquid scintillation counter.

Anti-HIV Activity in Fresh Human Cells: Assay in Fresh Human Monocyte-Macrophages

For isolation of adherent cells, 3 x 10^6 non-PHA stimulated peripheral blood cells were resuspended in Hanks buffered saline (with calcium and magnesium) supplemented with 10% human AB serum. The cells were placed in a 24-well microtiter plate at 37°C for 2 hours. Non-adherent cells were removed by vigorously washing six times. The adherent cells were cultured for 7 days in RPMI 1640 tissue culture medium with 15% fetal bovine serum. The cultures were carefully monitored for confluency during this incubation period. Infection of the cells was performed with the monocytotropic HIV-1 strains BaL or ADA and the matched pair of AZT-sensitive and AZT-resistant virus isolates. Each of these virus isolates was obtained from the NIAID AIDS Research and Reference Reagent Program. High titer pools of each of these viruses have been harvested from infected cultures of peripheral blood adherent cells and frozen in 1.0 ml aliquots at -80°C. Monocyte-macrophage monolayers were infected at an MOI of 0.1. Compounds to be evaluated in the monocyte-macrophage assay are added to the monolayers shortly before infection in order to

maximize the potential for identifying active compounds.

At 2 days post-infection, the medium was decanted and the cultures washed twice with complete medium in order to remove excess virus. Fresh medium alone or medium containing the appropriate concentrations of drugs was added and incubation continued for an additional 5 days. XTT-tetrazolium or trypan blue exclusion assays (for cell viability) and HIV p24 ELISA assays (for production of p24 core antigen) were performed on Day 7 post-infection. ELISA kits were purchased from Coulter. The assay is performed according to the manufacturer's recommendations. Control curves are generated in each assay to accurately quantify the amount of capsid protein in each sample. Data was obtained by spectrophotometric analysis at 450 nm using a Molecular Devices Vmax plate reader. P24 concentrations were calculated from the optical density values by use of the Molecular Device software package Soft Max.

SUB B24

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------ To determine the relative effect of inverted hybrid or inverted chimeric structure on oligonucleotide-mediated depletion of complement, the following experiments were performed. Venous blood was collected from healthy adult human volunteers. Serum was prepared for hemolytic complement assay by collecting blood into

vacutainers (Becton Dickinson #6430 Franklin Lakes, NJ) without commercial additives. Blood was allowed to clot at room temperature for 30 minutes, chilled on ice for 15 minutes, then centrifuged at 4°C to separate serum. Harvested serum was kept on ice for same day assay or, alternatively, stored at -70°C. Buffer, or an oligonucleotide sample was then incubated with the serum. The oligonucleotides tested were 25mer oligonucleotide phosphodiesters or phosphorothioates, 25mer hybrid oligonucleotides, 25mer inverted hybrid oligonucleotides, 25mer chimeric oligonucleotides, and 25mer inverted chimeric oligonucleotides. Representative hybrid oligonucleotides were composed of seven to 13 2-O-methyl ribonucleotides flanked by two regions of six to nine deoxyribonucleotides each. Representative 25mer inverted hybrid oligonucleotides were composed of 17 deoxyribonucleotides flanked by two regions of four ribonucleotides each. Representative 25mer chimeric oligonucleotides were composed of six methylphosphonate deoxyribonucleotides and 19 phosphorothioate deoxyribonucleotides. Representative inverted chimeric oligonucleotides were composed of from 16 to 17 phosphorothioate deoxyribonucleotides flanked by regions of from two to seven methylphosphonate deoxyribonucleotides, or from six to eight methylphosphonate deoxyribonucleotides flanked by nine to ten phosphorothioate deoxyribonucleotides, or two phosphorothioate regions ranging from two to 12 oligonucleotides, flanked by three phosphorothioate regions ranging in size from two

SUB B24 Cont'd

to six nucleotides in length. A standard CH50 assay (See Kabat and Mayer (eds), *Experimental Immunochemistry*, 2d Ed., Springfield, IL, CC Thomas, p. 125) for complement-mediated lysis of sheep red blood cells (Colorado Serum Co.) sensitized with anti-sheep red blood cell antibody (hemolysin, Diamedix, Miami, FL) was performed, using duplicate determinations of at least five dilutions of each test serum, then hemoglobin release into cell-free supernates was measured spectrophotometrically at 541 nm.

SUB B24 CON5

EXAMPLE 3

In Vitro Mitogenicity Studies

To determine the relative effect of inverted hybrid or inverted chimeric structure on oligonucleotide-mediated mitogenicity, the following experiments were performed. Spleen was taken from a male CD1 mouse (4-5 weeks, 20-22 g; Charles River, Wilmington, MA). Single cell suspensions were prepared by gently mincing with frosted edges of glass slides. Cells were then cultured in RPMI complete media (RPMI media supplemented with 10% fetal bovine serum (FBS), 50 micromolar 2-mercaptoethanol (2-ME), 100 U/ml penicillin, 100 micrograms/ml streptomycin, 2 mM L-glutamine). To minimize oligonucleotide degradation, FBS was first heated for 30 minutes at 65°C (phosphodiester-containing oligonucleotides) or 56°C (all other oligonucleotides). Cells were plated in 96 well dishes at 100,000 cells per well (volume of 100 microliters/well). One type of each

oligonucleotide described in Example 2 above in 10 microliters TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) was added to each well. After 44 hours of culturing at 37°C, one microcurie tritiated thymidine (Amersham, Arlington Heights, IL) was added in 20 microliters RPMI media for a 4 hour pulse labelling. The cells were then harvested in an automatic cell harvester (Skatron, Sterling, VA) and the filters were assessed using a scintillation counter. In control experiments for mitogenicity, cells were treated identically, except that either media (negative control) or concanavalin A (positive control) was added to the cells in place of the oligonucleotides.

All of the inverted hybrid oligonucleotides proved to be less immunogenic than phosphorothioate oligonucleotides. Inverted hybrid oligonucleotides having phosphodiester linkages in the 2'-O-methyl region appeared to be slightly less immunogenic than those containing phosphorothioate linkages in that region. No significant difference in mitogenicity was observed when the 2'-O-methyl ribonucleotide region was pared down from 13 to 11 or to 9 nucleotides. Inverted chimeric oligonucleotides were also generally less mitogenic than phosphorothioate oligonucleotides. In addition, these oligonucleotides appeared to be less mitogenic than traditional chimeric oligonucleotides, at least in cases in which the traditional chimeric oligonucleotides had significant numbers of methylphosphonate linkages near the 3' end. Increasing the number of

methylphosphonate linkers in the middle of the oligonucleotide from 5 to 6 or 7 did not appear to have a significant effect on mitogenicity. These results indicate that incorporation of inverted hybrid or inverted chimeric structure into an oligonucleotide can reduce its mitogenicity.

EXAMPLE 4

In Vitro Studies

To determine the relative effect of inverted hybrid or inverted chimeric structure on oligonucleotide-induced mitogenicity, the following experiments were performed. Venous blood was collected from healthy adult human volunteers. Plasma for clotting time assay was prepared by collecting blood into siliconized vacutainers with sodium citrate (Becton Dickinson #367705), followed by two centrifugations at 4°C to prepare platelet-poor plasma. Plasma aliquots were kept on ice, spiked with various test oligonucleotides described in Example 2 above, and either tested immediately or quickly frozen on dry ice for subsequent storage at -20°C prior to coagulation assay. Activated partial thromboplastin time (aPTT) was performed in duplicate on an Electra 1000C (Medical Laboratory Automation, Mount Vernon, NY) according to the manufacturer's recommended procedures, using Actin FSL (Baxter Dade, Miami, FL) and calcium to initiate clot formation, which was measured photometrically. Prolongation of aPTT was taken as an indication of clotting inhibition side effect produced by the oligonucleotide.

Traditional phosphorothioate oligonucleotides produced the greatest prolongation of aPTT, of all of the oligonucleotides tested. Traditional hybrid oligonucleotides produced somewhat reduced prolongation of aPTT. In comparison with traditional phosphorothioate or traditional hybrid oligonucleotides, all of the inverted hybrid oligonucleotides tested produced significantly reduced prolongation of aPTT. Inverted hybrid oligonucleotides having phosphodiester linkages in the 2'-O-substituted ribonucleotide region had the greatest reduction in this side effect, with one such oligonucleotide having a 2'-O-methyl RNA phosphodiester region of 13 nucleotides showing very little prolongation of aPTT, even at oligonucleotide concentrations as high as 100 micrograms/ml. Traditional chimeric oligonucleotides produce much less prolongation of aPTT than do traditional phosphorothioate oligonucleotides. Generally, inverted chimeric oligonucleotides retain this characteristic. At least one inverted chimeric oligonucleotide, having a methylphosphonate region of seven nucleotides flanked by phosphorothioate regions of nine nucleotides, gave better results in this assay than the traditional chimeric oligonucleotides at all but the highest oligonucleotide concentrations tested. These results indicate that inverted hybrid and inverted chimeric oligonucleotides may provide advantages in reducing the side effect of clotting inhibition when they are administered to modulate gene expression *in vivo*.

EXAMPLE 5

In Vivo Complement Activation Studies

Rhesus monkeys (4-9 kg body weight) are acclimatized to laboratory conditions for at least 7 days prior to the study. On the day of the study, each animal is lightly sedated with ketamine-HCl (10 mg/kg) and diazepam (0.5 mg/kg). Surgical level anesthesia is induced and maintained by continuous ketamine intravenous drip throughout the procedure. The oligonucleotides described in Example 2 above are dissolved in normal saline and infused intravenously via a cephalic vein catheter, using a programmable infusion pump at a delivery rate of 0.42 mg/minute. For each oligonucleotide, doses of 0, 0.5, 1, 2, 5 and 10 mg/kg are administered to two animals each over a 10 minute infusion period. Arterial blood samples are collected 10 minutes prior to oligonucleotide administration and 2, 5, 10, 20, 40 and 60 minutes after the start of the infusion, as well as 24 hours later. Serum is used for determining complement CH50, using the conventional complement-dependent lysis of sheep erythrocyte procedure (see Kabat and Mayer, 1961, *supra*). At the highest dose, phosphorothioate oligonucleotide causes a decrease in serum complement CH50 beginning within 5 minutes of the start of infusion. Inverted hybrid and chimeric oligonucleotides are expected to show a much reduced or undetectable decrease in serum complement CH50 under these conditions.

EXAMPLE 6

In Vivo Mitogenicity Studies

CD1 mice are injected intraperitoneally with a dose of 50 mg/kg body weight of oligonucleotide described in Example 2 above. Forty-eight hours later, the animals are euthanized and the spleens are removed and weighed. Animals treated with inverted hybrid or inverted hybrid oligonucleotides are expected to show no significant increase in spleen weight, while those treated with oligonucleotide phosphorothioates are expected to show modest increases in spleen weight.

EXAMPLE 7

In Vivo Clotting Studies

Rhesus monkeys are treated as in Example 5. From the whole blood samples taken, plasma for clotting assay is prepared, and the assay performed, as described in Example 4. It is expected that prolongation of aPTT will be substantially reduced for both inverted hybrid oligonucleotides and for inverted chimeric oligonucleotide, relative to traditional oligonucleotide phosphorothioates.

EXAMPLE 8

RNase H Activity Studies

To determine the ability of inverted hybrid oligonucleotides and inverted chimeric oligonucleotides to activate RNase H when bound to a complementary RNA molecule, the following experiments were performed. Each type of oligonucleotide described in Example 2 above was incubated together with a molar equivalent quantity of complimentary oligoribonucleotide (0.266 micromolar concentration of each), in a cuvette containing a final volume of 1 ml RNase H buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 M KCl, 2% glycerol, 0.1 mM DTT). The samples were heated to 95°C, then cooled gradually to room temperature to allow annealing to form duplexes. Annealed duplexes were incubated for 10 minutes at 37°C, then 5 units RNase H was added and data collection commenced over a three hour period. Data was collected using a spectrophotometer (GBC 920, GBC Scientific Equipment, Victoria, Australia) at 259 nm. RNase H degradation was determined by hyperchromic shift.

As expected, phosphodiester oligonucleotides behaved as very good co-substrates for RNase H-mediated degradation of RNA, with a degradative half-life of 8.8 seconds. Phosphorothioate oligonucleotides produced an increased half-life of 22.4 seconds. Introduction of a 2'-O-methyl ribonucleotide segment at either end of the oligonucleotide further worsened RNase H activity (half-life = 32.7 seconds). In contrast,

introducing a 2'-O-methyl segment into the middle of the oligonucleotide (inverted hybrid structure) always resulted in improved RNase H-mediated degradation. When a region of 13 2'-O-methylribonucleoside phosphodiesters was flanked on both sides by phosphorothioate DNA, the best RNase H activity was observed, with a half-life of 7.9 seconds. Introduction of large blocks of methylphosphonate-linked nucleosides at the 3' end of the oligonucleotide either had no effect or caused further deterioration of RNase H activity even when in a chimeric configuration. Introduction of methylphosphonate linked nucleosides at the 5' end, however, improved RNase H activity, particularly in a chimeric configuration with a single methylphosphonate linker at the 3' end (best half-life = 8.1 seconds). All inverted chimeric oligonucleotides with methylphosphonate core regions flanked by phosphorothioate regions gave good RNase results, with a half-life range of 9.3 to 14.4 seconds. These results indicate that the introduction of inverted hybrid or inverted chimeric structure into phosphorothioate-containing oligonucleotides can restore some or all of the ability of the oligonucleotide to act as a co-substrate for RNase H, a potentially important attribute for an effective antisense agent.

EXAMPLE 9

Melting Temperature Studies

To determine the effect of inverted hybrid or inverted chimeric structure on stability of the duplex formed between an antisense oligonucleotide

and a target molecule, the following experiments were performed. Thermal melting (Tm) data were collected using a spectrophotometer (GBC 920, GBC Scientific Equipment, Victoria, Australia), which has six 10 mm cuvettes mounted in a dual carousel. In the Tm experiments, the temperature was directed and controlled through a peltier effect temperature controller by a computer, using software provided by GBC, according to the manufacturer's directions. Tm data were analyzed by both the first derivative method and the mid-point method, as performed by the software. Tm experiments were performed in a buffer containing 10 mM PIPES, pH 7.0, 1 mM EDTA, 1 M NaCl. A refrigerated bath (VWR 1166, VWR, Boston, MA) was connected to the peltier-effect temperature controller to absorb the heat. Oligonucleotide strand concentration was determined using absorbance values at 260 nm, taking into account extinction coefficients.

EXAMPLE 10

Tumor Growth and Antisense Treatment

LS-174T human colon carcinoma cells (1 x 10^6 cells) were inoculated subcutaneously (s.c.) into the left flank of athymic mice. A single dose of RI_α antisense hybrid (Oligo 164, SEQ ID NO:4), inverted hybrid (Oligo 166, SEQ ID NO:6), or inverted chimeric (Oligo 190, SEQ ID NO:1) oligonucleotides or control oligonucleotide (Oligo 169, SEQ ID NO:7); Oligo 168 (SEQ ID NO:5); Oligo 188, SEQ ID NO:3)) as shown in Table 1 (1 mg per 0.1 ml saline per mouse), or saline (0.1 ml per

mouse), was injected s.c. into the right flank of mice when tumor size reached 80 to 100 mg, about 1 week after cell inoculation. Tumor volumes were obtained from daily measurement of the longest and shortest diameters and calculation by the formula, $4/3\pi r^3$ where r = (length + width)/4. At each indicated time, two animals from the control and antisense-treated groups were killed, and tumors were removed and weighed. The results are shown in FIG. 1. These results show that the size of the tumor in the animal treated with the inverted hybrid oligonucleotide 166 having SEQ ID NO:6 was surprisingly smaller from three days after injection onward than the phosphorothioate oligonucleotide 164 having SEQ ID NO:1. That this effect was sequence-specific is also demonstrated in FIG. 1: control oligonucleotide 168 (SEQ ID NO:3) has little ability to keep tumor size at a minimum relative to the hybrid and inverted hybrid oligonucleotides.

EXAMPLE 11

Photoaffinity Labelling and
Immunoprecipitation of RI_α Subunits

The tumors are homogenized with a Teflon/glass homogenizer in ice-cold buffer 10 (Tris-HCl, pH 7.4, 20 mM; NaCl, 100 mM; NP-40, 1%; sodium deoxycholate, 0.5%; $MgCl_2$, 5 mM; pepstatin, 0.1 mM; antipain, 0.1 mM; chymostatin, 0.1 mM; leupeptin, 0.2 mM; aprotinin, 0.4 mg/ml; and soybean trypsin inhibitor, 0.5 mg/ml; filtered through a 0.45-μm pore size membrane), and

centrifuged for 5 min in an Eppendorf microfuge at 4°C. The supernatants are used as tumor extracts.

The amount of PKA RI_α subunits in tumors is determined by photoaffinity labelling with $8\text{-}N_3\text{-}[^{32}P]$cAMP followed by immunoprecipitation with RI_α antibodies as described by Tortora et al. *(Proc. Natl. Acad. Sci. (USA)* (1990) **87:**705-708). The photoactivated incorporation of $8\text{-}N_3\text{-}[^{32}P]$cAMP (60.0 Ci/m-mol), and the immunoprecipitation using the anti-RI_α or anti-RII_β antiserum and protein A Sepharose and SDS-PAGE of solubilized antigen-antibody complex follows the method previously described (Tortora et al. (1990) *Proc. Natl. Acad. Sci. (USA)* **87:**705-708; Ekanger et al. (1985) *J. Biol. Chem.* **260:**3393-3401). It is expected that the amount of RI_α in tumors treated with hybrid, inverted hybrid, and inverted chimeric oligonucleotides of the invention will be reduced compared with the amount in tumors treated with mismatch, straight phosphorothioate, or straight phosphodiester oligonucleotide controls, saline, or other controls.

EXAMPLE 12

cAMP-Dependent Protein Kinase Assays

Extracts (10 mg protein) of tumors from antisense-, control antisense-, or saline-treated animals are loaded onto DEAE cellulose columns (1 x 10 cm) and fractionated with a linear salt gradient (Rohlff et al. (1993) *J. Biol. Chem.* **268:**5774-5782). PKA activity is determined in the

absence or presence of 5 μM cAMP as described below (Rohlff et al. (1993) *J. Biol. Chem.* **268:**5774-5782). cAMP-binding activity is measured by the method described previously and expressed as the specific binding (Tagliaferri et al. (1988) *J. Biol. Chem.* **263:**409-416).

After two washes with Dulbecco's phosphate-buffered saline, cell pellets (2 x 10^6 cells) are lysed in 0.5 ml of 20 mM Tris (pH 7.5), 0.1 mM sodium EDTA, 1 mM dithiothreitol, 0.1 mM pepstatin, 0.1 mM antipain, 0.1 mM chymostatin, 0.2 mM leupeptin, 0.4 mg/ml aprotinin, and 0.5 mg/ml soybean trypsin inhibitor, using 100 strokes of a Dounce homogenizer. After centrifugation (Eppendorf 5412) for 5 min, the supernatants are adjusted to 0.7 mg protein/ml and assayed (Uhler et al. (1987) *J. Biol. Chem.* **262:**15202-15207) immediately. Assays (40 μl total volume) are performed for 10 min at 300°C and contained 200 μM ATP, 2.7 x 10^6 cpm γ[^{32}P]ATP, 20 mM $MgCl_2$, 100 μM Kemptide (Sigma K-1127) (Kemp et al. (1977) *J. Biol. Chem.* **252:**4888-4894), 40 mM Tris (pH 7.5), ± 100 μM protein kinase inhibitor (Sigma P-3294) (Cheng et al. (1985) *Biochem. J.* **231:**655-661), ± 8 μM cAMP and 7 μg of cell extract. The phosphorylation of Kemptide is determined by spotting 20 μl of incubation mixture on phosphocellulose filters (Whatman, P81) and washing in phosphoric acid as described (Roskoski (1983) *Methods Enzymol.* **99:**3-6). Radioactivity is measured by liquid scintillation using Econofluor-2 (NEN Research Products NEF-969). It is expected that PKA and cAMP binding

activity will be reduced in extracts of tumors treated with the hybrid, inverted hybrid, and inverted chimeric oligonucleotides of the invention.

EXAMPLE 13

EFFECT OF HYB 165 WITH DOCETAXEL AND MONOCLONAL ANTIBODY MAb C225 ON THE GROWTH OF ZR75-1 HUMAN BREAST CANCER CELLS

MATERIALS AND METHODS

Materials.

HYB 165, a 18-mer mixed backbone oligonucleotides (MBO) targeted against the N-terminal 8-13 codons of the human RIα regulatory subunit of PKA, synthesized by the procedure previously described was provided by Hybridon Inc., Cambridge, MA. The antisense used had the following sequence: HYB 165, ***GCGU***GCCTCCTCAC***UGGC*** and contains 2-O-methyl-modified ribonucleotide bases (bold italics) at the 5' and 3' ends and unmodified oligodeoxynucleotide bases in the middle. Docetaxel was a kind gift from Rhone Poulenc Rorer, Origgio, Italy, and used after dilution in appropriate solvent as 100x concentrated stock. The monoclonal antibody MAb C225 is a human-mouse chimeric IgG_1 that binds to the EGFR, competes with natural ligands for receptor binding and blocks the EGFR tyrosine kinase activation. Clinical grade MAb C225 was kindly provided by Dr. H. Waksal, ImClone Systems, New York, NY.

Cell lines.

ZR75-1 human breast cancer cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in DMEM medium supplemented with 10% heat-inactivated FBS, 20 mM HEPES, pH 7.4, penicillin (100 UI/ml), streptomycin (100 μg/ml) and 4 mM glutamine (ICN, Irvine, UK) in a humified atmosphere of 95% air and 5% CO_2 at 37° C.

Soft agar growth.

Cells (10^4 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson) and treated with various

concentrations of docetaxel (day 0). HYB 165 and C225 were added together after 12 hrs (day 1) and on day 3. Twelve days after the last treatment, cells were stained with nitroblue tetrazolium (Sigma) and colonies larger than 0.05 mm were counted.
Experiments were performed twice in triplicate.

Results

HYB 165 0.1 μM, which alone causes about 8% inhibition and C225 0.25 μg/ml, which alone causes about 8% inhibition, were added to ZR75-1 cells treated with docetaxel 0.01 nM, which alone causes less than 12% inhibition, determining an average 93% inhibition. See Figure 2.

Conclusions

HYB 165, MAb C225 and docetaxel, at the low inhibitory doses of 0.1 μM, 0.25 μg/ml and 0.01 nM, respectively, cooperatively inhibit the growth of ZR75-1 cells when used in combination.

Figure Legend

Effect of the combination of Hyb 165, the MAb C225 and Docetaxel on the soft agar growth of ZR-75-1 breast cancer cells. The doses of the different agents are: HYB 165, 0.1 and 0.5 μM; Docetaxel, 0.01 nM; MAb C225, 0.25 μg/ml.
Data are expressed as percentage growth inhibition in reference to the growth of untreated control cells. The height of the bars on the left represents the sum of the individual agents effects and the expected percentage growth inhibition if drugs are additive when used in combination. The total height of the solid bar indicates the actual observed growth inhibition when drugs were used in combination. Therefore, the differences between the heights of the paired bars reflect the magnitude of synergism of growth inhibition.
The data represent means and standard errors of triplicate determinations of two experiments.

EXAMPLE 14

EFFECT OF HYB 508 WITH DOCETAXEL AND MONOCLONAL ANTIBODY MAb C225 ON THE GROWTH OF ZR75-1 HUMAN BREAST CANCER CELLS

MATERIALS AND METHODS

Materials.

HYB 508, a 18-mer mixed backbone oligonucleotides (MBO) targeted against the N-terminal 8-13 codons of the human RIα regulatory subunit of PKA, synthesized by the procedure previously described was provided by Hybridon Inc., Cambridge, MA. The antisense used had the following sequence: HYB 508, ***GCAU***GCTTCCACAC***AGGC*** and contains 2-O-methyl-modified ribonucleotide bases (bold italics) at the 5' and 3' ends and unmodified oligodeoxynucleotide bases in the middle. HYB 508 is a control oligonucleotide of HYB 165, containing four mismatched nucleotides (underlined). Docetaxel was a kind gift from Rhone Poulenc Rorer, Origgio, Italy, and used after dilution in appropriate solvent as 100x concentrated stock. The monoclonal antibody MAb C225 is a human-mouse chimeric IgG_1 that binds to the EGFR, competes with natural ligands for receptor binding and blocks the EGFR tyrosine kinase activation. Clinical grade MAb C225 was kindly provided by Dr. H. Waksal, ImClone Systems, New York, NY.

Cell lines.

ZR75-1 human breast cancer cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in DMEM medium supplemented with 10% heat-inactivated FBS, 20 mM HEPES, pH 7.4, penicillin (100 UI/ml), streptomycin (100 µg/ml) and 4 mM glutamine (ICN, Irvine, UK) in a humified atmosphere of 95% air and 5% CO_2 at 37° C.

Soft agar growth.

Cells (10^4 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson) and treated with various concentrations of docetaxel (day 0). The HYB 508 and C225 were added together after 12 hrs (day 1) and on day 3. Twelve days after the last treatment, cells were stained with nitroblue tetrazolium (Sigma) and colonies larger than 0.05 mm were counted.

Experiments were performed twice in triplicate.

Results

HYB 508 0.5 μM, which alone causes about 6% inhibition and C225 0.25 μg/ml, which alone causes about 8% inhibition, were added to ZR75-1 cells treated with docetaxel 0.01 nM, which alone causes about 12% inhibition, determining an average 26% inhibition. See Figure 3.

Conclusions

HYB 508, MAb C225 and docetaxel, at the low inhibitory doses of 0.5 μM, 0.25 μg/ml and 0.01 nM, respectively, showed no cooperative antiproliferative effect on the growth of ZR-75-1 cells when used in combination.

Figure Legend

Effect of the combination of Hyb 508, the MAb C225 and Docetaxel on the soft agar growth of ZR-75-1 breast cancer cells. The doses of the different agents are: HYB 508, 0.5 μM; Docetaxel, 0.01 nM; MAb C225, 0.25 μg/ml.

Data are expressed as percentage growth inhibition in reference to the growth of untreated control cells. The height of the bars on the left represents the sum of the individual agents effects and the expected percentage growth inhibition if drugs are additive when used in combination. The total height of the solid bar indicates the actual observed growth inhibition when drugs were used in combination. Therefore, the differences between the heights of the paired bars reflect the magnitude of synergism of growth inhibition.

The data represent means and standard errors of triplicate determinations of two experiments.

EXAMPLE 15

EFFECT OF HYB 165 WITH OR WITHOUT PACLITAXEL ON THE GROWTH OF GEO HUMAN COLON CANCER CELLS

MATERIALS AND METHODS

Materials.

HYB 165, a 18-mer mixed backbone oligonucleotides (MBO) targeted against the N-terminal 8-13 codons of the human RIα regulatory subunit of PKA, synthesized by the procedure previously described was provided by Hybridon Inc., Cambridge, MA. The antisense used had the following sequence: HYB 165, ***GCGU***GCCTCCTCAC***UGGC*** and contains 2-O-methyl-modified ribonucleotide bases (bold italics) at the 5' and 3' ends and unmodified oligodeoxynucleotide bases in the middle. Paclitaxel was purchased from Sigma (St Louis, MO) and used after dilution in appropriate solvent as 100x concentrated stock.

Cell lines.

GEO human colon cancer cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in McCoy medium supplemented with 10% heat-inactivated FBS, 20 mM HEPES, pH 7.4, penicillin (100 UI/ml), streptomycin (100 µg/ml) and 4 mM glutamine (ICN, Irvine, UK) in a humified atmosphere of 95% air and 5% CO_2 at 37° C.

Soft agar growth.

Cells (10^4 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson) and treated with various concentrations of paclitaxel (day 0). The HYB 165 was added after 12 hrs (day 1) and on day 2, 3 and 4. 12 days after the last treatment, cells were stained with nitroblue tetrazolium (Sigma) and colonies larger than 0.05 mm were counted.

Experiments were performed twice in triplicate.

Results

A dose-dependent effect of paclitaxel at doses ranging between 0.1 and 10 nM was observed, determining up to about 60% growth inhibition. HYB 165 0.5 μM, which alone causes about 20% inhibition, was added to GEO cells treated with *a*) paclitaxel 1 nM, which alone causes less than 5% inhibition, determining an average 40% inhibition; *b*) paclitaxel 5 nM, which alone causes about 20% inhibition, determining an average 62% inhibition; *c*) paclitaxel 10 nM, which alone causes abot 58% inhibition, determining an average 86% inhibition. See Figure 4.

Conclusions

HYB 165 at the low inhibitory dose of 0.5 μM cooperatively inhibit the growth of GEO cells when used in a sequential combination with different doses of paclitaxel.

EXAMPLE 16

EFFECT OF HYB 165 AND ITS CONTROL HYB 508 ON THE GROWTH OF 1A9PTX22 HUMAN OVARIAN CANCER CELLS

MATERIALS AND METHODS

Materials. 18-mer mixed backbone oligonucleotides (MBO) targeted against the N-terminal 8-13 codons of the human RIα regulatory subunit of PKA, synthesized by the procedure previously described were provided by Hybridon Inc., Cambridge, MA. The antisense used had the following sequences: HYB 165, *GCGU*GCCTCCTCAC*UGGC*; HYB 508, *GCAU*GCTTCCACAC*AGGC*. HYB 165 and HYB 508 are chimeric compounds containing 2-O-methyl-modified ribonucleotide bases (bold italics) at the 5' and 3' ends and unmodified oligodeoxynucleotide bases in the middle. HYB 508 is a control oligo containing four mismatched nucleotides as underlined.

Cell lines. The 1A9PTX22 cell line, a paclitaxel (PTX)-resistant clone of the human ovarian carcinoma cell line 1A9, was isolated by exposing 1A9 cells to 5 ng/ml PTX in the presence of 5 μg/ml verapamil, a P glicoprotein antagonist. 1A9PTX22 cells were kindly provided by Dr. Giannakakou, NCI Bethesda, MD, USA. Cells were maintained in RPMI medium supplemented with 10% heat-inactivated FBS, 20 mM HEPES, pH 7.4 penicillin (100 UI/ml), streptomycin (100 μg/ml) and 4 mM glutamine (ICN, Irvine, UK) 15 ng/ml PTX and 5 μg/ml verapamil in a humified atmosphere of 95% air and 5% CO_2 at 37° C. 7 days before experiments were performed, PTX and verapamil were removed from culture medium.

Soft agar growth. Cells (10^4 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson) and treated with various concentrations of HYB 165 or HYB508 every 48 hours for three times. After 12 days the cells were stained with nitroblue tetrazolium (Sigma, St. Louis, MO) and colonies larger than 0.05 mm were counted.

Experiments were performed twice in triplicate.

RESULTS

Two different 18-mer MBO complementary to the RIα subunit of PKAI sequence, HYB 165 and its control oligomer HYB 508, differing only in 4 nucleotide bases, were tested to study their effect on soft agar growth of 1A9 human ovarian cancer cells. While HYB 165 determined a dose-dependent inhibition of colony formation at doses ranging between 0.1 and 2.5 μM in all cell lines, the HYB 508 control sequence showed a modest or no growth inhibitory effect. HYB 165 determined an inhibition of 1A9PTX22 cell growth of approximately 5% at a dose of 0.1 μM, of about 50% at 0.5 μM, of about 82% at 1μM and achieved over 95% at 2.5 μM (Fig. 2). Conversely, HYB 508 caused a growth inhibition which at the highest dose of 2.5 μM achieved 10%.
See Figure 5.

SUB B25

CONCLUSIONS

HYB 165 causes a dose-dependent growth inhibitory effect on 1A9PTX22 cells, while its mismatched control oligomer causes a modest growth inhibitory effect (no more than 10%).

EXAMPLE 17

EFFECT OF HYB 165 AND ITS CONTROL HYB 508 ON THE GROWTH OF 1A9PTX10 HUMAN OVARIAN CANCER CELLS

MATERIALS AND METHODS

Materials. 18-mer mixed backbone oligonucleotides (MBO) targeted against the N-terminal 8-13 codons of the human RIα regulatory subunit of PKA, synthesized by the procedure previously described were provided by Hybridon Inc., Cambridge, MA. The antisense used had the following sequences: HYB 165, ***GCGU***GCCTCCTCAC***UGGC***; HYB 508, ***GCAU***GCTTCCACAC***AGGC***. HYB 165 and HYB 508 are chimeric compounds containing 2-O-methyl-modified ribonucleotide bases (bold italics) at the 5' and 3' ends and unmodified oligodeoxynucleotide bases in the middle. HYB 508 is a control oligo containing four mismatched nucleotides as underlined.

Cell lines. The 1A9PTX10 cell line, a paclitaxel (PTX)-resistant clone of the human ovarian carcinoma cell line 1A9, was isolated by exposing 1A9 cells to 5 ng/ml PTX in the presence of 5 μg/ml verapamil, a P glicoprotein antagonist. 1A9PTX10 cells were kindly provided by Dr. Giannakakou, NCI Bethesda, MD, USA. Cells were maintained in RPMI medium supplemented with 10% heat-inactivated FBS, 20 mM HEPES, pH 7.4, penicillin (100 UI/ml), streptomycin (100 μg/ml) and 4 mM glutamine (ICN, Irvine, UK) 15 ng/ml PTX and 5 μg/ml verapamil in a humified atmosphere of 95% air and 5% CO_2 at 37° C. 7 days before experiments were performed, PTX and verapamil were removed from culture medium.

Soft agar growth. Cells (10^4 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson) and treated with various concentrations of HYB 165 or HYB508 every 48 hours for three times. After 12 days the cells were stained with nitroblue tetrazolium (Sigma, St. Louis, MO) and colonies larger than 0.05 mm were counted.

Experiments were performed twice in triplicate.

RESULTS

Two different 18-mer MBO complementary to the RIα subunit of PKAI sequence, HYB 165 and its control oligomer HYB 508, differing only in 4 nucleotide bases, were tested to study their effect on soft agar growth of 1A9 human ovarian cancer cells. While HYB 165 determined a dose-dependent inhibition of colony formation at doses ranging between 0.1 and 2.5 μM in all cell lines, the HYB 508 control sequence showed a modest or no growth inhibitory effect. HYB 165 determined an inhibition of 1A9PTX10 cell growth of approximately 5% at a dose of 0.1 μM, of about 43% at 0.5 μM, of about 70% at 1μM and achieved over 85% at 2.5 μM (Fig. 2). Conversely, HYB 508 caused a growth inhibition which at the highest dose of 2.5 μM achieved 10%. See Figure 6.

CONCLUSIONS

HYB 165 causes a dose-dependent growth inhibitory effect on 1A9PTX10 cells, while its mismatched control oligomer causes a modest growth inhibitory effect (no more than 10%).

EXAMPLE 18

EFFECT OF HYB 165 AND ITS CONTROL HYB 508 ON THE GROWTH OF 1A9 HUMAN OVARIAN CANCER CELLS

MATERIALS AND METHODS

Materials. 18-mer mixed backbone oligonucleotides (MBO) targeted against the N-terminal 8-13 codons of the human RIα regulatory subunit of PKA, synthesized by the procedure previously described were provided by Hybridon Inc., Cambridge, MA. The antisense used had the following sequences: HYB 165, *GCGU*GCCTCCTCAC*UGGC*; HYB508, *GCAU*GCTTCCACAC*AGGC*. HYB 165 and HYB 508 are chimeric compounds containing 2-O-methyl-modified ribonucleotide bases (bold italics) at the 5' and 3' ends and unmodified oligodeoxynucleotide bases in the middle. HYB 508 is a control oligo containing four mismatched nucleotides as underlined.

Cell lines. The 1A9 cell line is a clone of the human ovarian carcinoma cell line, A2780. 1A9 cells were kindly provided by Giannakakou, NCI Bethesda, MD, USA. Cells were maintained in RPMI medium supplemented with 10% heat-inactivated FBS, 20 mM HEPES, pH 7.4 penicillin (100 UI/ml), streptomycin (100 μg/ml) and 4 mM glutamine (ICN, Irvine, UK) in a humified atmosphere of 95% air and 5% CO_2 at 37° C.

Soft agar growth. Cells (10^4 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson) and treated with various concentrations of HYB 165 or HYB508 every 48 hours for three times. After 12 days the cells were stained with nitroblue tetrazolium (Sigma, St. Louis, MO) and colonies larger than 0.05 mm were counted.

Experiments were performed twice in triplicate.

RESULTS

Two different 18-mer MBOs complementary to the RIα subunit of PKAI sequence, HYB 165 and its control oligomer HYB 508, differing only in 4 nucleotide bases, were stidied to evaluate their effect on soft agar growth of 1A9 human ovarian cancer cells. While HYB 165 determined a dose-dependent inhibition of colony formation at doses ranging between 0.1 and 2.5 μM in all cell lines, the HYB 508 control sequence showed a modest or no growth inhibitory effect. HYB 165 determined an inhibition of 1A9 cell growth of approximately 5% at a dose of 0.1 μM, of about 41% at 0.5 μM, of about 90% at 1μM and achieved over 95% at 2.5 μM (Fig. 2). Conversely, HYB 508 caused a growth inhibition which at the highest dose of 2.5 μM achieved 20% inhibition. See Figure 7.

CONCLUSIONS

HYB 165 causes a dose-dependent growth inhibitory effect on 1A9 cells, while its mismatched control oligomer causes a modest growth inhibitory effect (no more than 20%).

EXAMPLE 19

EFFECT OF HYB 508 WITH OR WITHOUT MONOCLONAL ANTIBODY Mab C225 ON THE GROWTH OF ZR-75-1 HUMAN BREAST CANCER CELLS

MATERIALS AND METHODS

Materials. HYB 508, a 18-mer mixed backbone oligonucleotides (MBO) targeted against the N-terminal 8-13 codons of the human RIα regulatory subunit of PKA, synthesized by the procedure previously described was provided by Hybridon Inc., Cambridge, MA. The antisense used had the following sequence: HYB 508, ***GCAU***GCTTCCACAC***AGGC*** and contains 2-O-methyl-modified ribonucleotide bases (bold italics) at the 5' and 3' ends and unmodified oligodeoxynucleotide bases in the middle. HYB 508 is a control oligonucleotide of HYB 165, containing four mismatched nucleotides (underlined). The monoclonal antibody Mab C225 is a human-mouse chimeric IgG_1 that binds to the EGFR, competes with natural ligands for receptor binding and blocks the EGFR tyrosine kinase activation. Clinical grade MAbC225 was kindly provided by Dr. H. Waksal, ImClone Systems, New York, NY.

Cell lines. ZR-75-1 human breast cancer cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in DMEM medium supplemented with 10% heat-inactivated FBS, 20 mM HEPES, pH 7.4, penicillin (100 UI/ml), streptomycin (100 μg/ml) and 4 mM glutamine (ICN, Irvine, UK) in a humified atmosphere of 95% air and 5% CO_2 at 37° C.

Soft agar growth. Cells (10^4 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson) and treated with various concentrations of Mab C225 and/or of HYB508 every 48 hours for three times.After 12 days the cells were stained with nitroblue tetrazolium (Sigma) and colonies larger than 0.05 mm were counted.

Experiments were performed twice in triplicate.

RESULTS

HYB 508 0.5 μM (i-l), which alone causes about 5% inhibition of ZR-75-1 cell growth, was used in combination with *i*) Mab C225 0.25 μg/ml, which alone causes about 10% inhibition, determining an average 12% inhibition; *j*) Mab C225 0.5 μg/ml, which alone causes about 47% inhibition, determining an average 45% inhibition; *k*) Mab C225 1 μg/ml, which alone causes about 68% inhibition, determining an average 77% inhibition; *l*) Mab C225 2.5 μg/ml, which alone causes about 76% inhibition, determining an average 82% inhibition. See Figure 8.

CONCLUSIONS

HYB 508 at the dose of 0.5 μM showed no cooperative antiproliferative effect on the growth of ZR-75-1 cells when used in combination with different doses of Mab C225.

Figure Legend

Effect of the combination of two different agents on the growth of ZR-75-1 breast cancer cells. HYB 508 0.5 μM (i-l) in combination with MAb C225 0.25 μg/ml,i), 0.5 μg/ml (j), 1 μg/ml (k) and 2.5 μg/ml (l).

Data are expressed as percentage growth inhibition in reference to the growth of untreated control cells. The height of the bars on the left represents the sum of the individual agents effects and the expected percentage growth inhibition if drugs are additive when used in combination. The total height of the solid bar indicates the actual observed growth inhibition when drugs were used in combination. Therefore, the differences between the heights of the paired bars reflect the magnitude of synergism of growth inhibition.

The data represent means and standard errors of triplicate determination of at least two experiments.

EXAMPLE 20

EFFECT OF HYB 165 AND HYB 618 ON THE GROWTH OF OVCAR-3 OVARIAN CANCER CELLS

MATERIALS AND METHODS

Materials. 18-mer mixed backbone oligonucleotides (MBO) targeted against the N-terminal 8-13 codons of the human RIα regulatory subunit of PKA, synthesized by the procedure previously described were provided by Hybridon Inc., Cambridge, MA. The antisense used had the following sequences: HYB 165, *GCGU*GCCTCCTCAC*UGGC*; HYB618, *GCAU*GCATCCGCAC*AGGC*. HYB 165 and HYB 618 are chimeric compounds containing 2-O-methyl-modified ribonucleotide bases (bold italics) at the 5' and 3' ends and unmodified oligodeoxynucleotide bases in the middle. HYB 618 is a control oligo containing four mismatched nucleotides as underlined.

Cell lines. OVCAR human ovarian cancer cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in DMEM and HAM'S F-12 (1:1) supplemented with 10% heat-inactivated FBS, 20 mM HEPES, pH 7.4, penicillin (100 UI/ml), streptomycin (100 μg/ml) and 4 mM glutamine (ICN, Irvine, UK) in a humified atmosphere of 95% air and 5% CO_2 at 37° C.

Soft agar growth. Cells (10^4 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson) and treated with various concentrations of HYB 165 or HYB295 every 48 hours for three times. After 12 days the cells were stained with nitroblue tetrazolium (Sigma, St. Louis, MO) and colonies larger than 0.05 mm were counted.

Experiments were performed twice in triplicate.

Results

Two different 18-mer MBO complementary to the RIα subunit of PKAI sequence, HYB 165 and its control oligomer HYB 618, differing only in 4 nucleotide bases, were tested to study their effect on soft agar growth of GEO human colon cancer cells. While HYB 165 determined a dose-dependent inhibition of colony formation at doses ranging between 0.1 and 2.5 μM in all cell lines, the HYB 618 control sequence showed a modest or no growth inhibitory effect. HYB 165 determined an inhibition of OVCAR-3 cell growth of approximately 25% at a dose of 0.1 μM, of about 58% at 0.5 μM, of about 75% at 1 μM and about 95% at 2.5 μM (Fig. 2). Conversely, HYB 618 caused a growth inhibition which at the highest dose of 2.5 μM achieved 15%. See Figure 9.

Conclusions

HYB 165 causes a dose-dependent growth inhibitory effect on OVCAR-3 cells, while its mismatched control oligomer causes a modest growth inhibitory effect (less than 15%).

EXAMPLE 21

EFFECT OF HYB 165 WITH OR WITHOUT DOCETAXEL ON THE GROWTH OF ZR75-1 HUMAN BREAST CANCER CELLS

MATERIALS AND METHODS

Materials. HYB 165, a 18-mer mixed backbone oligonucleotides (MBO) targeted against the N-terminal 8-13 codons of the human RIα regulatory subunit of PKA, synthesized by the procedure previously described was provided by Hybridon Inc., Cambridge, MA. The antisense used had the following sequence: HYB 165, ***GCGU***GCCTCCTCAC***UGGC*** and contains 2-O-methyl-modified ribonucleotide bases (bold italics) at the 5' and 3' ends and unmodified oligodeoxynucleotide bases in the middle. Docetaxel was a kind gift from Rhone Poulenc Rorer, Origgio, Italy, and used after dilution in appropriate solvent as 100x concentrated stock.

Cell lines. ZR75-1 human breast cancer cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in DMEM medium supplemented with 10% heat-inactivated FBS, 20 mM HEPES, pH 7.4, penicillin (100 UI/ml), streptomycin (100 µg/ml) and 4 mM glutamine (ICN, Irvine, UK) in a humified atmosphere of 95% air and 5% CO_2 at 37° C.

Soft agar growth. Cells (10^4 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson) and treated with various concentrations of docetaxel (day 0). The HYB 165 was added after 12 hrs (day 1) and on day 3. Twelve days after the last treatment, cells were stained with nitroblue tetrazolium (Sigma) and colonies larger than 0.05 mm were counted.
Experiments were performed twice in triplicate.

Results

A dose-dependent effect of docetaxel at doses ranging between 0.01 and 0.3 nM was observed, determining up to about 80% growth inhibition. HYB 165 0.1(a-d) μM, which alone causes about 5% inhibition, was added to ZR75-1 cells treated with *a*) docetaxel 0.01 nM, which alone causes less than 15% inhibition, determining an average 40% inhibition; *b*) docetaxel 0.03 nM, which alone causes about 40% inhibition, determining an average 70% inhibition; *c*) docetaxel 0.1 nM, which alone causes about 72% inhibition, determining an average 86% inhibition; *d*) docetaxel 0.3 nM, which alone causes about 85% inhibition, determining an average 97%.

HYB 165 0.5 μM(e-f), which alone causes about 15% inhibition, was added to ZR75-1 cells treated with *e*) docetaxel 0.01 nM, which alone causes less than 15% inhibition, determining an average 65% inhibition; *f*) docetaxel 0.03 nM, which alone causes about 40% inhibition, determining an average 66% inhibition; *g*) docetaxel 0.1 nM, which alone causes about 72% inhibition, determining an average 86% inhibition; *h*) docetaxel 0.3 nM, which alone causes about 85% inhibition, determining an average 99% inhibition. See Figure 10.

Conclusions

HYB 165 at the low inhibitory doses of 0.1 μM and 0.5 μM cooperatively inhibits the growth of ZR75-1 cells when used in a sequential combination with different doses of docetaxel.

Figure Legend

Effect of the combination of two different agents on the growth of ZR-75-1 breast cancer cells. HYB 165 0.1 μM (a-d) and 0.5 μM (e-f) in combination with Docetaxel 0.01 nM (a-e); 0.03 nM (b-f); 0.1 nM (c-g); 0.3 nM (d-h).

Data are expressed as percentage growth inhibition in reference to the growth of untreated control cells. The height of the bars on the left represents the sum of the individual agents effects and the expected percentage growth inhibition if drugs are additive when used in combination. The total height of the solid bar indicates the actual observed growth inhibition when drugs were used in combination. Therefore, the differences between the heights of the paired bars reflect the magnitude of synergism of growth inhibition.

The data represent means and standard errors of triplicate determination of at least two experiments.

EXAMPLE 22

EFFECT OF HYB 508 WITH OR WITHOUT DOCETAXEL ON THE GROWTH OF ZR-75-1 HUMAN BREAST CANCER CELLS

MATERIALS AND METHODS

Materials. HYB 508, a 18-mer mixed backbone oligonucleotides (MBO) targeted against the N-terminal 8-13 codons of the human RIα regulatory subunit of PKA, synthesized by the procedure previously described was provided by Hybridon Inc., Cambridge, MA. The antisense used had the following sequence: HYB 508, ***GCAU***GCTTCCACAC***AGGC*** and contains 2-O-methyl-modified ribonucleotide bases (bold italics) at the 5' and 3' ends and unmodified oligodeoxynucleotide bases in the middle. HYB 508 is a control oligonucleotide of HYB 165, containing four mismatched nucleotides (underlined). Docetaxel was a kind gift from Rhone Poulenc Rorer, Origgio, Italy, and used after dilution in appropriate solvent as 100x concentrated stock.

Cell lines. ZR-75-1 human breast cancer cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in DMEM medium supplemented with 10% heat-inactivated FBS, 20 mM HEPES, pH 7.4, penicillin (100 UI/ml), streptomycin (100 µg/ml) and 4 mM glutamine (ICN, Irvine, UK) in a humified atmosphere of 95% air and 5% CO_2 at 37° C.

Soft agar growth. Cells (10^4 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson) and treated with various concentrations of docetaxel (day 0).The HYB 508 was added after 12 hrs and on day 2, 3 and 4. After 12 days the cells were stained with nitroblue tetrazolium (Sigma) and colonies larger than 0.05 mm were counted.

Experiments were performed twice in triplicate.

RESULTS

A dose-dependent effect of docetaxel at doses ranging between 0.01 and 0.3 nM was observed, determining up to about 80% growth inhibition. HYB508 0.5 μM (i-l), which alone causes about 7% inhibition, was added to ZR75-1 cells treated with cells treated with : *i*) docetaxel 0.01 nM, which alone causes less than 15% inhibition, determining an average 20% inhibition; *j*) docetaxel 0.03 nM, which alone causes about 40% inhibition, determining an average 42% inhibition; *k*) docetaxel 0.1 nM, which alone causes about 72% inhibition, determining an average 78% inhibition; l) docetaxel 0.3 nM, which alone causes about 85% inhibition, determining an average 82%. See Figure 11.

Conclusions

HYB 508 at the dose of 0.5 μM showed no cooperative antiproliferative effect on the growth of ZR-75-1 cells when used in a sequential combination with different doses of docetaxel.

Figure Legend

Effect of the combination of two different agents on the growth of ZR-75-1 breast cancer cells. HYB 508 0.5 μM (i-l) in combination with Docetaxel 0.01 nM (i); 0.03 nM (j); 0.1 nM (k); 0.3 nM (l).

Data are expressed as percentage growth inhibition in reference to the growth of untreated control cells. The height of the bars on the left represents the sum of the individual agents effects and the expected percentage growth inhibition if drugs are additive when used in combination. The total height of the solid bar indicates the actual observed growth inhibition when drugs were used in combination. Therefore, the differences between the heights of the paired bars reflect the magnitude of synergism of growth inhibition.

The data represent means and standard errors of triplicate determination of at least two experiments.

EXAMPLE 23

EFFECT OF HYB 165 WITH OR WITHOUT MONOCLONAL ANTIBODY MAb C225 ON THE GROWTH OF ZR-75-1 HUMAN BREAST CANCER CELLS

MATERIALS AND METHODS

Materials. HYB 165, a 18-mer mixed backbone oligonucleotides (MBO) targeted against the N-terminal 8-13 codons of the human RIα regulatory subunit of PKA, synthesized by the procedure previously described was provided by Hybridon Inc., Cambridge, MA. The antisense used had the following sequence: HYB 165, ***GCGU***GCCTCCTCAC***UGGC,*** and contains 2-O-methyl-modified ribonucleotide bases (bold italics) at the 5' and 3' ends and unmodified oligodeoxynucleotide bases in the middle. The monoclonal antibody MAb C225 is a human-mouse chimeric IgG_1 that binds to the EGFR, competes with natural ligands for receptor binding and blocks the EGFR tyrosine kinase activation. Clinical grade Mab C225 was kindly provided by Dr. H. Waksal, ImClone Systems, New York, NY.

Cell lines. ZR-75-1 human breast cancer cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in DMEM medium supplemented with 10% heat-inactivated FBS, 20 mM HEPES, pH 7.4, penicillin (100 UI/ml), streptomycin (100 μg/ml) and 4 mM glutamine (ICN, Irvine, UK) in a humified atmosphere of 95% air and 5% CO_2 at 37° C.

Soft agar growth. Cells (10^4 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson) and treated with various concentrations of Mab C225 and/or of HYB165 every 48 hours for three times. After 12 days the cells were stained with nitroblue tetrazolium (Sigma) and colonies larger than 0.05 mm were counted.

Experiments were performed twice in triplicate.

RESULTS

HYB 165 0.1 μM (a-d), which alone causes about 2% inhibition of ZR-75-1 cell growth, was used in combination with *a*) Mab C225 0.25 μg/ml, which alone causes about 10% inhibition, determining an average 37% inhibition; *b*) Mab C225 0.5 μg/ml, which alone causes about 47% inhibition, determining an average 65% inhibition; *c*) Mab C225 1 μg/ml, which alone causes about 68% inhibition, determining an average 85% inhibition; *d*) Mab C225 2.5 μg/ml, which alone causes about 76% inhibition, determining an average 90% inhibition.

HYB 165 at the higher dose of 0.5 μM (e-h), which alone causes about 10% inhibition of ZR-75-1 cell growth, was used in combination with *e*) Mab C225 0.25 μg/ml, which alone causes about 10% inhibition, determining an average 57% inhibition; f) Mab C225 0.5 μg/ml, which alone causes about 47% inhibition, determining an average 70% inhibition; *g*) Mab C225 1 μg/ml, which alone causes about 68% inhibition, determining an average 90% inhibition; *h*) Mab C225 2.5 μg/ml, which alone causes about 76% inhibition, determining an average 98% inhibition. See Figure 12.

CONCLUSIONS

HYB 165 at the low inhibitory dose of 0.1 μM and 0.5 μM cooperatively inhibit the growth of ZR-75-1 cells when when used in combination with different doses of Mab C225.

Figure Legend

Effect of the combination of two different agents on the growth of ZR-75-1 breast cancer cells. HYB 165 0.1 μM (a-d) and 0.5 μM (e-f) or HYB 508 0.5 μM (i-l) in combination with MAb C225 0.25 μg/ml (a,e,i), 0.5 μg/ml (b,f,j), 1 μg/ml (c,g,k) and 2.5 μg/ml (d,h,l).

Data are expressed as percentage growth inhibition in reference to the growth of untreated control cells. The height of the bars on the left represents the sum of the individual agents effects and the expected percentage growth inhibition if drugs are additive when used in combination. The total height of the solid bar indicates the actual observed growth inhibition when drugs were used in combination. Therefore, the differences between the heights of the paired bars reflect the magnitude of synergism of growth inhibition.

The data represent means and standard errors of triplicate determination of two experiments.

EXAMPLE 24

EFFECT OF HYB 165 AND HYB 295 ON THE GROWTH OF ZR-75-1 HUMAN BREAST CANCER CELLS

MATERIALS AND METHODS

Materials. 18-mer mixed backbone oligonucleotides (MBO) targeted against the N-terminal 8-13 codons of the human RIα regulatory subunit of PKA, synthesized by the procedure previously described were provided by Hybridon Inc., Cambridge, MA. The antisense used had the following sequences: HYB 165, ***GCGU***GCCTCCTCAC***UGGC***; HYB295, ***GCAU***GCATCCGCAC***AGGC***. HYB 165 and HYB 295 are chimeric compounds containing 2-O-methyl-modified ribonucleotide bases (bold italics) at the 5' and 3' ends and unmodified oligodeoxynucleotide bases in the middle. HYB 295 is a control oligo containing four mismatched nucleotides as underlined.

Cell lines. ZR-75-1 human breast cancer cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in DMEM medium supplemented with 10% heat-inactivated FBS, 20 mM HEPES, pH 7.4, penicillin (100 UI/ml), streptomycin (100 µg/ml) and 4 mM glutamine (ICN, Irvine, UK) in a humified atmosphere of 95% air and 5% CO_2 at 37° C.

Soft agar growth. Cells (10^4 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson) and treated with various concentrations of HYB 165 or HYB295 every 48 hours for three times. After 12 days the cells were stained with nitroblue tetrazolium (Sigma, St. Louis, MO) and colonies larger than 0.05 mm were counted.

Experiments were performed twice in triplicate.

RESULTS

Two different 18-mer MBO complementary to the RIα subunit of PKAI sequence, HYB 165 and its control oligomer HYB 295, differing only in 4 nucleotide bases, were tested to study their effect on soft agar growth of ZR-75-1 human breast cancer cells. While HYB 165 determined a dose-dependent inhibition of colony formation at doses ranging between 0.1 and 2.5 μM in all cell lines, the HYB 295 control sequence showed a modest or no growth inhibitory effect. HYB 165 determined an inhibition of ZR-75-1 cell growth of approximately 5% at a dose of 0.1 μM, of about 34% at 1 μM and achieved over 85% at 2.5 μM (Fig. 2). Conversely, HYB 295 caused a growth inhibition which at the highest dose of 2.5 μM achieved 10%. See Figure 13.

CONCLUSIONS

HYB 165 causes a dose-dependent growth inhibitory effect on ZR-75-1 cells, while its mismatched control oligomer causes a modest growth inhibitory effect (no more than 10%).

EXAMPLE 25

EFFECT OF HYB 165 AND HYB 508 ON THE GROWTH OF ZR-75-1 HUMAN BREAST CANCER CELLS

MATERIALS AND METHODS

Materials.

18-mer mixed backbone oligonucleotides (MBO) targeted against the N-terminal 8-13 codons of the human RIα regulatory subunit of PKA, synthesized by the procedure previously described were provided by Hybridon Inc., Cambridge, MA. The antisense used had the following sequences: HYB 165, *GCGU*GCCTCCTCAC*UGGC*; HYB 508, *GCAU*GCTTCCACAC*AGGC*. HYB 165 and HYB 508 are chimeric compounds containing 2-O-methyl-modified ribonucleotide bases (bold italics) at the 5' and 3' ends and unmodified oligodeoxynucleotide bases in the middle. HYB 508 is a control oligo containing four mismatched nucleotides as underlined.

Cell lines.

ZR-75-1 human breast cancer cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in DMEM medium supplemented with 10% heat-inactivated FBS, 20 mM HEPES, pH 7.4, penicillin (100 UI/ml), streptomycin (100 µg/ml) and 4 mM glutamine (ICN, Irvine, UK) in a humified atmosphere of 95% air and 5% CO_2 at 37° C.

Soft agar growth. Cells (10^4 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson) and treated with various concentrations of HYB 165 or HYB508 every 48 hours for three times. After 12 days the cells were stained with nitroblue tetrazolium (Sigma, St. Louis, MO) and colonies larger than 0.05 mm were counted.

Experiments were performed twice in triplicate.

Results

Two different 18-mer MBO complementary to the RIα subunit of PKAI sequence, HYB 165 and its control oligomer HYB 508, differing only in 4 nucleotide bases, were tested to study their effect on soft agar growth of ZR-75-1 human breast cancer cells. While HYB 165 determined a dose-dependent inhibition of colony formation at doses ranging between 0.1 and 2.5 μM in all cell lines, the HYB 508 control sequence showed a modest or no growth inhibitory effect. HYB 165 determined an inhibition of ZR-75-1 cell growth of approximately 5% at a dose of 0.1 μM, of about 34% at 1 μM and achieved over 85% at 2.5 μM (Fig. 2). Conversely, HYB 508 caused a growth inhibition which at the highest dose of 2.5 μM achieved 10%. See Figure 14.

Conclusions

HYB 165 causes a dose-dependent growth inhibitory effect on ZR-75-1 cells, while its mismatched control oligomer causes a modest growth inhibitory effect (no more than 10%).

EXAMPLE 26

EFFECT OF HYB 165 AND HYB 295 ON THE GROWTH OF GEO COLON CANCER CELLS

MATERIALS AND METHODS

Materials. 18-mer mixed backbone oligonucleotides (MBO) targeted against the N-terminal 8-13 codons of the human RIα regulatory subunit of PKA, synthesized by the procedure previously described were provided by Hybridon Inc., Cambridge, MA. The antisense used had the following sequences: HYB 165, ***GCGU***GCCTCCTCAC***UGGC***; HYB295, ***GCAU***GC<u>A</u>TCC<u>G</u>CAC***AGGC***. HYB 165 and HYB 295 are chimeric compounds containing 2-O-methyl-modified ribonucleotide bases (bold italics) at the 5' and 3' ends and unmodified oligodeoxynucleotide bases in the middle. HYB 295 is a control oligo containing four mismatched nucleotides as underlined.

Cell lines. GEO human colon cancer cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in McCoy's Medium 5A supplemented with 10% heat-inactivated FBS, 20 mM HEPES, pH 7.4, penicillin (100 UI/ml), streptomycin (100 µg/ml) and 4 mM glutamine (ICN, Irvine, UK) in a humified atmosphere of 95% air and 5% CO_2 at 37° C.

Soft agar growth. Cells (10^4 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson) and treated with various concentrations of HYB 165 or HYB295 every 48 hours for three times. After 12 days the cells were stained with nitroblue tetrazolium (Sigma, St. Louis, MO) and colonies larger than 0.05 mm were counted. See Figure 15.

Experiments were performed twice in triplicate.

EXAMPLE 27

HYB 165 inhibits tumor growth after i.p. or oral administration

We investigated the antitumor activity of HYB 165 (AS RIα) in nude mice bearing GEO colon cancer xenograts, using either the intraperitoneal (i.p.) or the oral route of administration. When established GEO tumors of approximately 0.2 cm^3 were detectable, groups of 10 mice were treated i.p. with either HYB 165 or a control modified backbone oligonucleotide with a scrambled sequence, at 5 or 10 mg/kg/dose, daily on days 7 to 11 and 14 to 18. Figure 16A shows that i.p. administration of HYB 165 caused a dose-dependent inhibition of growth up to 40% at a dose of 10 mg/kg/dose. The control oligonucleotide produced no inhibition at 10 mg/kg/dose.

Following oral administration, modified backbone oligonucleotides (MBOs) are absorbed in the upper and lower part of the GI tract and distributed to major organs (S. Agrawal and R. Zhang, In: Antisense Research and Application, S. T. Crooke, ed.), Handbook of Experimental Pharmacology, Springer, Berlin, p. 525-543 (1998). Therefore, HYB 165 and the control oligonucleotide were administered to GEO tumor-bearing mice as described above, except that HYB 165 and the control oligonucleotide were administered orally. As shown in Figure 16B, at a dose of 10 mg/kg/dose, the two cycles of treatment with HYB 165 caused an average inhibition of tumor growth of about 60% as compared to untreated mice, while the tumor size of the mice treated with the

control scramble oligonucleotide was only slightly affected.

SUB B27 CONT

EXAMPLE 28

Oral HYB 165 cooperatively inhibits tumor growth and increases survival in combination with taxol.

On day 7 after tumor cell injection, one group of 10 mice was treated with taxol (20 mg/kg/dose, i.p.), and the treatment was repeated every 2 weeks (on day 21 and day 35) for a total of three cycles. Two other groups of mice were treated with either HYB 165 (AS RIα) or a control MBO with a scrambled sequence (10 mg/kg/dose, p.o.), daily for five days (days 8-12). Treatment was repeated every 2 weeks (days 22-26 and days 36-40) for a total of three cycles. Two more groups of mice were treated with taxol and either HYB 165 or the control MBO, administering the taxol (20 mg/kg/dose, i.p.) on day 7, followed by oral administration of either HYB 165 or the control MBO daily for five days (days 8-12). The sequential treatment was repeated with the same schedule every 2 weeks for a total of three cycles.

As illustrated in Figure 17A, treatment with either taxol or the HYB 165 alone inhibited tumor growth as compared to control untreated mice or to mice treated with the scramble MBO. HYB 165 was more effective than taxol, causing over 50% inhibition of tumor size at the completion of the three cycles of treatment. However, shortly after the end of treatment, GEO tumors resumed the growth rate of those in untreated mice or in mice

SUB B27

treated with the scramble MBO. When taxol and HYB 165 were used in combination, a marked and sustained inhibition of tumor growth was observed. In fact, tumors of mice treated with taxol and HYB 165 grew very slowly for approximately 60 days following the end of treatment, at which time they resumed a faster growth rate (Figure 17A). Administration of the scramble MBO in combination with taxol produced an effect simiolar to that of taxol alone. Within approximately 5 weeks, GEO tumors reached a size not compatible with normal life in all untreated mice and in mice treated with the scramble MBO (Figure 17B). A slight increase in survival time was observed in the group treated with taxol alone, an effect similar to that observed in mice treated with taxol followed by the scramble MBO (data not shown). Treatment with HYB 165 alone also increased survival time as compared to the control group. The delayed GEO tumor growth observed in the group treated with taxol in combination with HYB 165 was accompanied by a prolonged mice life span, when, when analyzed with the log-rank test (N. Mantel, *Cancer Chem. Rep.*, 163-170 (1966)), was significantly different as compared to controls ($P < 0.0001$), to the taxol-treated group ($P < 0.0001$) or to the group treated with scramble MBO plus taxol ($P < 0.0001$). In fact, the only mice still alive at 10 weeks after tumor cell injection were those treated with the combination of taxol and HYB 165. Furthermore, about 50% of the mice in this group were still alive after 15 weeks. The combined treatment with taxol and HYB 165 was well

SUB B2] CONT

SUB B27 cont

tolerated, since no weight loss or other signs of acute or delayed toxicity were observed.

EXAMPLE 29

SUB B28

Cooperative antitumor effect of HYB 165 with taxol is accompanied by inhibition of new vessels formation and growth factors production.

SUB B29

Tumor specimens from the different groups of mice were examined by histochemical analysis at different time points to evaluate the expression of a variety of biological parameters. Resuts of the analysis performed on tumor specimens after two cycles of treatment are presented in Table I. Treatment with HYB 165 inhited expression of the target RIα protein in the tumor. This effect was further increased with HYB 165 was used in combination with taxol. No other treatment was able to affect RIα expression. These results suggest that inhibition of RIα expression is not dependent on growth inhibition.

TGFα and AR are growth factors which bind to EGFR and control human colon cancer growth through autocrine and paracrine mechanisms (F. Ciardiello and G. Tortora, *Clin. Cancer Res.* 4:821-828 (1998); D. S. Salomon, *Crit. Rev. Oncol. Hematol.* 19:183-232 (1995)). Unlike taxol, treatment with HYB 165 inhibited the expression of TGF α and AR. Inhibition of AR was further enhanced when taxol was used in combination with HYB 165. Moreover, the combination of taxol and HYB 165 almost completely suppressed cell proliferation, as demonstrated by Ki67 staining.

Loda *et al.* (*Nature Medicine* 3:231-234 (1997)) discloses that the cyclin-dependent kinase (CDK) inhibitor p27 is directly related to cell entry into S phase and proliferation and that reduction of its expression correlates with poor prognosis in colon cancer patients. Unlike taxol, HYB 165 alone is able to increase p27 expression. Moreover, a 2.5-fold increase in intensely positive cell staining for p27 was observed in the tumor samples from mice treated with taxol and antisense RIα.

In recent years, the critical role of tumor-induced neovascularization in neoplastic development, progression and metastasis has been elucidated (J. I. Fokman, In: J. Mendelsohn *et al.*, eds., *The Molecular Basis of Cancer*, pp 206-232, Phaladelphia: WB Saunders (1995). A reliable histologic estimate of novel blood vessels on tumor specimens is the microvessel count (MVC) in the most intense areas of neovascularization. In the present study, tumor-induced neovascularization was quantified by immunohistochemistry using an anti-Factor VIII related antigen monoclonal antibody (N. Weidner, *Breast Cancer Res. Treat.*, 36:169-180 (1995)). As shown in Table I, a significant inhibition of staining was obtained with HYB 165 (about 80%) as well as with taxol (over 60%), as compared to samples from untreated mice or mice treated with the scramble MBO. Combined treatment with taxol and HYB 165 completely suppressed vessel formation in GEO tumors, demonstrating that the cooperative antitumor effect was associated with the marked inhibition of several factors controlling cell

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cycle, proliferation and angiogenesis of this human colon cancer model.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.